KAUNAS UNIVERSITY OF TECHNOLOGY

VAIDA KRAUJALIENĖ

BIOREFINING OF *BERGENIA CRASSIFOLIA* L., *FAGOPYRUM ESCULENTUM* MOENCH. AND *SOLIDAGO VIRGAUREA* L. BY HIGH PRESSURE EXTRACTION METHODS AND EVALUATION OF OBTAINED PRODUCTS

Doctoral Dissertation
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VAIDA KRAUJALIENĖ

STORALAPĖS BERGENIJOS (*BERGENIA CRASSIFOLIA* L.), GRIKIŲ ŽIEDYNŲ (*FAGOPYRUM ESCULENTUM* MOENCH.) BEI RYKŠTENĖS (*SOLIDAGO VIRGAUREA* L.) BIORAFINAVIMAS, TAIKANT AUKŠTO SLĖGIO EKSTRAKCIJOS METODUS, IR GAUTŲ PRODUKTŲ SAVYBIŲ ĮVERTINIMAS

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Abreviations

AA	Antioxidant activity;
AAPH	2,2'-Azobis(2-amidinopropane) dihydrochloride;
ABTS	2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid);
AC	Acetone;
BL	Bergenia leaf;
BR	Bergenia roots;
BWF	Buckwheat flowers;
CO_2–ET	Carbon dioxide Ethanol;
DW	Dry weight;
DWE	Dry weight extracts material;
DWP	Dry weight plant material;
EM	Emulsion;
ET	Ethanol;
ET/W	Ethanol/Water;
FC	Folin-Ciocalteu;
GA	Gallic acid;
GAE	Gallic acid equivalent;
GR	Goldenrod;
GRL	Goldenrod leaves;
HAT	Hydrogen atom transfer;
HPLC	High pressure liquid chromatography;
HX	Hexane;
IP	Induction period;
MeOH	Methanol;
ORAC	Oxygen radical absorbance capacity;
PBS	Phosphate buffered saline;
PF	Protection factor;
PLE	Pressurized liquid extraction;
RO	Rapeseed oil;
RSC	Radial scavenging capacity;
SFE	Supercritical fluid extraction;
TE	Trolox equivalent;
TEAC	Trolox equivalent entioxidant capacity;
TPC	Total phenolic content;
W	Water.

I. INTRODUCTION

1.1. Relevance of doctoral dissertation

Search, evaluation and application of natural antioxidants and other bioactive phytochemicals has become one of the most popular topics among food, agricultural and nutritional scientists due to the following main reasons:

(i) increasing evidence of health effects of numerous plant origin natural compounds;

(ii) rapid developments in functional foods, nutraceuticals, natural pharmaceuticals and cosmetics;

(iii) vast diversity of various under investigated species in the plant kingdom;

(iv) increasing consumer preferences for natural ingredients and additives in foods and other products;

(v) development of analytical techniques enabling fast and effective isolation and characterisation of natural compounds.

Medicinal and aromatic plants (MAP) are traditionally used in folk medicine as natural healing remedies with therapeutic effects such as adaptogenic, anticancer, antidiabetic, antihypertensive, anti–inflammatory, imunomodulating, antimicrobial, antiobesity, antioxidant, antitussive, antiviral, cerebro–protective, diuretic, gastro–protective, hepato–protective, skin whitening etc. The discovery of a very potent anti–malaria drug artemisinin, which was isolated from *Artemisia annua*, is one of the most exciting examples in the great success of natural product research (Tu, 2011). Pharmacological industry utilizes MAPs due to the presence of active chemical substances as agents for drug synthesis. They are valuable also for food and cosmetic industry as additives, due to their preservative effects because of the presence of antioxidants and antimicrobial constituents. To the worldwide known and commonly used medicinal plants with antioxidant activity belong botanicals from several families, especially Lamiaceae (rosemary, sage, oregano, marjoram, basil, thyme, mints, balm), Apiaceae (cumin, fennel, caraway), and Zingiberaceae (turmeric, ginger) (Škrovankova et al., 2012). Considering the huge biodiversity of aromatic plants a large part of them remains under-investigated. There is a lack for more comprehensive and systematic studies focused on phytochemicals evaluation and optimization of their processing into high added value ingredients, which could be applied for industrial prouction and utilization. For instance, the production of essential oils results in a very high content of residual by-products (up to 99.5% of the total plant material), which usually remain unused after distillation. Therefore in recent years search for effective extraction and fractionation methods, which would allow to employ whole plant material without generating any waste (such complex processess are called biorefining or in some cases agrorefining), became one of the most important gals. However, the most frequently used extraction methods in industry still are hydrodistillation, steam distillation and Soxhlet extraction using

organic solvents or their mixtures, which are often limited by long extraction time, high consumption of solvents, environmental pollution, thermal degradation of sensitive constituents during boiling, dissolving of some compounds in water during distillation of volatiles, difficulties in the removal of hazardous organic solvents after extraction, etc. (Reverchon et al., 2006). The new techniques for active compounds and other phytochemicals evaluation were adapted in few last decades, such as supercritical fluid, pressurized liquid, ultrasound and microwave assisted extractions as well as solid state fermentation. Development of combined techniques enlarges the opportunities for isolation and application of various antioxidants and other phytochemicals for foods, nutraceuticals, and cosmetics, medicinal and other purposes.

1.2. Aim and tasks of the work

The aim of the work was to develop multistep biorefining processes for extracting different *Bergenia crassifolia* L., *Fagopyrum esculentum* Moench. and *Solidago virgaurea* L. anatomical parts into functional ingredients by applying high pressure extraction and fractionation methods.

The following objectives were raised to achieve the aim:

1. To investigate various schemes of biorefining using high pressure extraction methods (SFE–CO_2 and PLE).
2. To evaluate the antioxidant properties of various fractions obtained by using different solvents by the selected *in vitro* assays and *in situ* (sunflower oil and oil/water emulsions).
3. To evaluate the antioxidant properties of extraction residues after each process step.
4. To determine phytochemical composition of various fractions by using chromatographic and spectroscopic methods.

1.3. Practical significance

The majority of the previously performed studies on the isolation of bioactive fractions and compounds from medicinal and aromatic plants have been applying conventional solvent extraction methods mainly using organic and often highly toxic solvents, such as acetone, methanol, ethyl acetate and others; moreover most of these studies have been performed for the analytical purposes. For practical purposes medicinal plants are usually extracted with hydroethanolic mixtures by using time-consuming maceration and percolation extraction methods. More comprehensive processing schemes of the selected plants as well as medicinal plants in general, intended to produce several valuable fractions of various phytochemical composition and functional properties, have not been investigated until now. The data may be used for the up-scaling the production of functional ingredients from selected plants to industrial levels and for preliminary prognosis of their uses and possible health benefits.

1.4. Scientific novelty

1. The application of the so-called biorefining concept to medicinal and other useful plants, which could be used for upscaling to pilot and industrial production, have not been reported previously.

2. To the best of our knowledge, multistep fractionation by using high pressure methods such as supercritical fluid extraction with carbon dioxide (SFE–CO_2) and pressurized liquid extraction (PLE) have not been applied for the isolation and fractionation of antioxidants and phytochemicals from the selected plants and any other medicinal plants previously.

1.5. Key points of the thesis

1. Application of biorefinery concept for multistep processing of the selected medicinal plants, namely *Bergenia crassifolia*, *Fagopyrum esculentum* and *Solidago virgaurea*, may provide valuable functional ingredients containing bioactive phytochemicals and antioxidatively active fractions.

1.6. Approbation of results

The results of the research have been published in three scientific articles in the journals indexed by CA WOS. The results of the research have also been presented in the 5 international scientific conferences.

1.7. Structure and content of the dissertation

The thesis is written in English. It consists of the list of abbreviations; introduction; literature review; materials and methods; results and discussion; conclusions; references; and list of publications relevant to the subject of the dissertation. The dissertation has 114 pages, 15 tables and 20 figures. The list of references includes 245 bibliographic sources.

II. LITERATURE REVIEW

2.1. Characterization of plants used in this study

2.1.1. Bergenia (*Bergenia crassifolia* L.)

Based on literature survey *B. crassifolia* (Saxifragaceae), common names – the badan, Siberian tea, Mongolian tea, leather bergenia, winter–blooming bergenia, heartleaf bergenia, elephant–ears, may be regarded as a rather scarcely studied plant species, particularly in terms of obtaining specific functional ingredients from different plant anatomical parts by their fractionation using various extraction methods, procedures and solvents. *B. crassifolia* is a perennial plant, native to central and eastern Asia, Siberia and the Altay Mountains in Russia, Mongolia and Xinjiang in China, while in Europe it is grown mainly as an ornamental plant. It is generally very frost–resistant and shade–tolerant, and can grow in extremely hard conditions such as well–drained stony slopes and rocks (Lu and Wang, 2003). The rhizomes and the leaves of this plant have been used for treating various disorders in the Asian folk medicine. Adaptogenic (Shikov et al., 2010) anti–inflammatory and immunomodulating (Lee and Kim, 2012 and Churin et al., 2005); antimicrobial (Kokoska et al., 2002), anticancer (Spiridonov et al., 2005) diuretic (Briukhanov and Fedoseeva, 1993); immunostimulating (Popov et al., 2005), antidiabetic (Kumar et al., 2012), antiviral (Tamura et al., 2010) and antioxidative effects (Hendrychová et al., 2014 and Ivanov et al., 2011) have been reported for *B. crasiffolia* leaves. For instance, water leaf extracts of *B. crassifolia* and *B.* × *ornate* were strong antioxidants in DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging assays (Hendrychová et al., 2014); *B. crassifolia* rhizomes strongly inhibited human pancreatic lipase activity *in vitro* (Ivanov et al., 2011), while ethanolic extracts of green leaves exhibited antioxidant properties in monitoring oxygen uptake rate in a gasometric system with 2,2′–azobisisobutyronitrile (AIBN)–initiated oxidation of isopropyl benzene (Shilova et al., 2006). More than 100 chemical components have been reported in *B. crassifolia*, including tannins, benzanoids (hydroquinone), flavonoids, polysaccharides, terpenes, aldehydes, etc. (Shikov et al., 2014), however bergenin, arbutin, hydroquinone, gallic, protocatechuic and ellagic acids were proposed as key compounds in leaf extracts (Shikov et al., 2010 and Shikov et al., 2012).

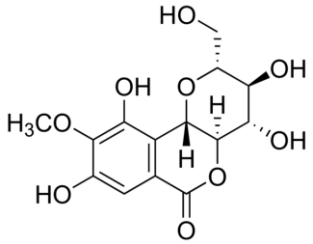


Fig. 2.1. Bergenin

Bergenin (glucoside of 4–O–methyl gallic acid) is one of active ingredients in herbal and Ayurvedic formulations used in folk medicine in Asia since 7th century. Firstly isolated from *Saxifraga siberica* (Bergenia) rhizomes exhibits antiviral, antifungal, antitussive, antiplasmodial, antiinflammatory, antihepatotoxic, antiarrhythmic, antitumor, antiulcerogenic, antidiabetic and wound healing properties (Bajracharya, 2015). The yields of this compound obtained from *Bergenia* species varried between 0.076 to 3.36 % (Uddin etal., 2013, Hay & Haynes, 1958). Other key compound of *B. crassifolia* is arbutin. It exists in plants in a free, ether or esterified form. Ussually it is biosynthesized by Ericaceae and Saxifragaceae species, but also is detected in other plant families including Asteraceae, Rosaceae, Lamiaceae and Apiaceae (Migas & Krauze–Baranowska, 2015). Hydroquinone as an aglycone of arbutin is responsible for antibacterial activity of plant extracts. It possesses astringent, disinfectant and antioxidant properties (Migas & Krauze–Baranowska, 2015). Plant extracts rich in arbutin fraction usually are used to treat urinary tract infections. Ellagic acid in bergenia plant varied between 24.8–65.6 mg/g methanolic and ethanolic extracts (Krasniewska et al., 2015). Various health effects have been reported for ellagic acid including antitumor activities in several cancer cells (Rocha et al., 2012, Wang et al., 2012, Radhika et al., 2012).

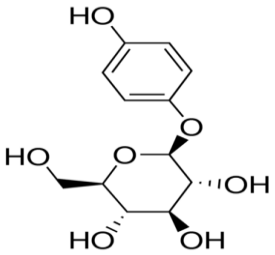


Fig. 2.2. Arbutin

2.1.2. Buckwheat (Fagopyrum esculentum)

Buckwheat is a Polygonaceae family gluten–free pseudocereal, which has been grown and used for food since ancient times. Common buckwheat (*Fagopyrum esculentum* Moench.) and tartary buckwheat (*F. tataricum* Gaertn.) are nowadays the most important commercially species. And although the cultivation of buckwheat declined sharply in the 20th century due to the adoption of nitrogen fertilizers that increased the productivity of other staples, nowadays it regains the popularity as a raw material for healthy foods. Bioactive compounds and biofunctional properties of buckwheat grain have been comprehensively reviewed: high quality proteins, resistant starch, dietary fiber, lignans, flavonoids, phytosterols, fagopyrins, fagopyritols, phenolic compounds, vitamins, minerals and antioxidants were reported as the vital constituents in buckwheat making them a highly valuable pseudocereal (Ahmed et al., 2014; Gimenez–Bastida, 2015; Zhang et al., 2012). Although grain–like seeds are the main edible part of buckwheat, phytochemical

composition and antioxidant properties of its leaves, stems, flowers, roots, hulls and sprouts have also been studied, mainly from the beginning of the 21st century (Acar et al., 2011; Bystricka et al., 2011; Dadakova & Kalinova, 2010; Hinneburg & Neubert, 2005; Holasova et al., 2002; Kim et al., 2008; Li et al., 2013; Liu et al., 2008; Peng et al., 2004; Quettier–Deleu et al., 2000; Sytar, 2014; Suzuki et al., 2009; Uddin et al., 2013; Zielinska et al., 2012). These studies resulted in the identification of various (poly)phenolics, mainly belonging to the classes of phenolic acids (ferulic, vanillic, chlorogenic, *p*–coumaric, *p*–anisic, salicylic, methoxycinnamic, vanillic, salicylic, p–anisic, 4–hydroxybenzoic, 4–hydroxy–3–methoxy benzoic), flavonoids (catechin, epicatechin, quercetin, kaempferol) and their glycosides (rutin, quercitrin) (Sytar, 2014; Sytar et al, 2014; Uddin et al., 2013).

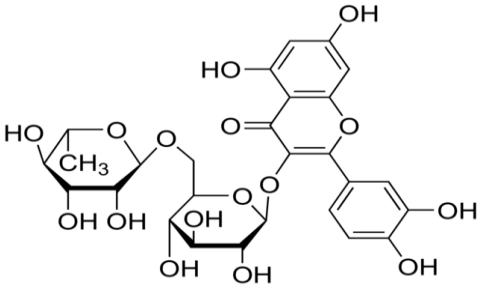


Fig. 2.3. Rutin

Squalene and α–tocopherol were reported in buckwheat leaves (Kalinova et al., 2006).

However, rutin was reported as the most abundant flavonoid in buckwheat; its content in leaves and flowers was remarkably higher than in seeds (Zielinska et al., 2012). Antioxidant properties of buckwheat inflorescences have also been reported in several articles (Acar et al., 2011; Bystricka et al., 2011; Hinneburg & Neubert, 2005; Holasova et al., 2001, 2002; Kim et al., 2008; Li et al., 2013; Liu et al., 2008; Quettier–Deleu et al., 2000; Sytar, 2014; Suzuki et al., 2009; Zielinska et al., 2012). For instance, Hinneburg & Neubert (2005) showed that the extract with good antioxidant activity, high content of phenolics, and low content of the phototoxic fagopyrin can be obtained by agitated maceration with 30% ethanol at 60 °C for 2 h.

Rutin (quercetin–3–O–rutinoside), the flavonol, is regarded as a vital nutritional component of the diet (Harborne, 1986); firstly it was identified in *Ruta graveolens*. It is found in reasonable quantities in buckwheat arial parts as well as in cereals. Most rutin is accumulated in the inflorescence (up to 12%, d.w.b. – dry weight basis), in stalks (0.4–1.0%, d.w.b.) and in upper leaves (8–10%, d.w.b.) (Hagels, 1999)). Among fruits, vegetables and grain crops, grapes and buckwheat are the most important rutin containing foods (Kreft et al., 2006). Rutin is responsible for the health benefits, including plasma cholesterol level reduction, neuroprotection, anti–inflammatory (Gimenez–Bastida & Zielinski, 2015), anticancer (Chen et al., 2013; Linet al., 2012), antidiabetic effects (Hsu et al., 2014), and improvement of hypertension conditions (Kim et al., 2009; Mendes–Junior et al., 2013; Ugusman et al., 2014,) as well as gastrointestinal system (Kalant et al., 2007, Abdel–Raheem, 2010). Other quercetin derivatives such as avicularin (quercetin–3–O–α–L–arabinofuranoside), hyperoside (quercetin–3–O–galactoside), quercitrin (quercetin–3–O–α–L–rhamnoside) were reported to be potential chemopreventive compounds (Shaheen Taj & Nagarajan,) and also linked with possitive effect in reducing the risks of cardiovascular deseases (Minotti et al., 2004, Wang et al., 2013). In addition, quercitrin reduced UVB–irradiated oxidative DNA damage, opoptosis, and inflammation (Yin, et al., 2013).

Phenolic acids (PA), mostly found in free form, are concentrated in buckwheat brans (Guo et al., 2012a,b). Guo et al., (2012a) revealed that the most qantitative PA in fine bran is *p*–hydroxybenzoic (up to 360 mg/100 g), followed by caffeic (38 mg/100 g), chlorogenic (21 mg/100 gbran), and protocatechuic acid (18 mg/100 g). In the hulls, protocatechuic acid is the most abundant (54 mg/100 g DW) (Guo et al., 2012a). Sprouting may be employed to increase the contents of PA such as chlorogenic acid (Kim et al., 2008). Sytar (2015) and Lee et al. (2014) reported that the content of chlorogenic acid deppending on buckwheat type and growing conditions in the sprouts was 2.30–4.37 mg/g DW, whereas in flowers remarkably lower, 0.03–0.17. mg/g DW. Beneficial effects of PA on human health has been widely documented, e.g. the effects of clorogenic acid are associated with its antioxidant (Upadhyay and Mohan Rao, 2013), antibacterial (Karunanidhi et al. 2013), anti–inflammatory (Liang and Kitts, 2016), antiplatelet and antithrombotic (Fuentes et al. 2014) activities; it also was shown to possess hypertensive propeties (Tom et al., 2016).

2.1.3. Golden rod (*Solidago virgaurea* L.)

Goldenrod (*Solidago virgaurea* L.) is a member of the genus *Solidago* (family Asteraceae), which includes about 120 species of North American wildflowers and more than a dozen species inhabiting South America, Europe and Asia. All species are herbaceous and most of them difficult to distinguish from one another. All goldenrods are late bloomers, flowering in late summer into the fall. They are common along the edges of moist forests, roadsides and meadows. Goldenrod (GR) was introduced to Europe in the 18th century as an ornamental plant and became invasive some 100 years later. It has been traditionally used to treat inflammations of the urinary tract. Several recent studies have revealed that the aerial parts of GR (leaves, stems and flowers) show antioxidant, antimicrobial, antibacterial, antifungal, anti–inflammatory, antihypertensive, antitumor, anti–adipogenetic, cardioprotective, spasmolytic and diuretic effects (Vonkruedener et al., 1995, Gross et al., 2002, Thiem & Goślińska 2002, Apáti et al., 2003, Goluart et al., 2007, Demir et al., 2009, Starks et al., 2010, Wang et al., 2011, Laurençon et al., 2013, Anzlovar & Koce, 2014; El–Tantawy, 2014; Deng et al., 2015, Jang et al., 2016). For instance, goldenrod leaf and stem powder demonstrated antioxidant activity in raw ground pork (Kim et al., 2013) and was applied as dietary fiber in sausages to improve their quality characteristics (Choe et al., 2011). Alcohol extract of *S. virgaurea* herb had slightly stronger antibacterial properties compared with lipophilic one; however hexane extract exhibited antimutagenic activity, which was not found for alcoholic extract (Kolodziej et al.,

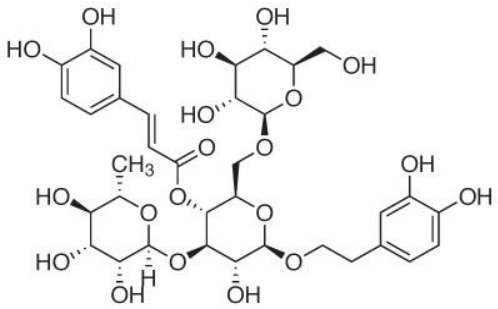


Fig. 2.4. Leiocarposide

2011). Antimicrobial, sedative, cytotoxic and hypotensive effects were also reported for the essential oils of *Solidago* species (Chanotiya & Yadav, 2008, Huang et al., 2012, Kołodziej et al., 2011 and Mishra et al., 2011).

Various poly(phenols) were identified in GR leaves and herb previously: phenolic acids (cafeic, caffeoylquinic, chlorogenic, gallic, rosmarinic, acids, cinnamic acid hydroxyl derivatives), flovonoids (kaemferol, isorhamnetin, leiocarposide, quercetin) and their derivatives (afzelin, astragalin, hyperoside, isoquercitrin, kaempferol–3–O–robinobioside, narcissin, nikotiflorin, quercitrin, rutin) (Pietta et al., 1991, Bader et al, 1998; Kristó et al., 2002, Apáti et al., 2002, 2003; Papp et al., 2004; Nugroho et al., 2009; Condrat et al., 2009, 2010; Jaiswal et al., 2011, Rosáon et al., 2014; Radušienė et al., 2015). Previous chemical investigations resulted in the isolation of flavonoid quercitrin as the main component of the aerial parts of *S. chilensis* (Gutierrez et al., 1981; Torres et al., 1987). Leiocarposide was recognised as a lead structure for the quality assurance of Solidaginis virgaureae herba (Bader et al., 1998).

2.2. Secondary metabolites in plants

Plant secondary metabolites (PSM) are natural bioactive agents, which are derived from primary metabolites. Plant kingdom produces more than 100,000 PSMs, which usually do not have any immediate effect in the survival of plants but have a long-term effect (Costa et al., 2012). PSMs can serve as (i) signal compounds (to attract pollinating and seed–dispersing animals or defend against herbivores and microbes); (ii) UV protectors, as antioxidants; (iii) odour, taste and colour compounds; (iv) mobile nitrogen stores. Therefore, PSMs have significant contribution in pharmaceutical, nutrition and cosmetic industries (Ramakrishna & Ravishankar, 2011; Fang et al., 2011). PSM metabolism is necessitate of various factors as genetic, ontogenetic, morphogenetic and enviromental.

Based on biosynthetic pathway, PSMs are classified into three major groups: terpenes (or isoprenoids), phenolic compounds (phenylpropanoids and flavonoids) and nitrogen containing compounds (alkaloids, glucosinolates and cyanogenic glycosides) (Fang et al., 2011). Further this section is mainly focused on phenolic compounds.

2.2.1. Terpenes

Terpenoids are one of the main groups of PSM. Based on five carbon unit (isoprene unit), terpenes are classified into different groups like monoterpenes (10 carbons or two C5 units), sesquiterpenes (three C5 units), diterpenes (four C5 units), triterpenes (six C5 units), tetraterpenes (eight C5 units) and polyterpenoids ([C5]n).

Mono and sesquiterpenes some volatile constituents of the essential oils of various plants, fruits and berries, containing only carbon, hydrogen and oxygen atoms. Some terpenoids like gibberellins (diterpenes), sterols (triterpenes), carotenoids (tetraterpenes), and abscisic acid (sesquiterpenes), play an important role in the growth of plants. (Verma & Shukla, 2015). Terpenes are synthesized in plants via two different pathways, one occurs in cytoplasm and other in plastids. The mevalonate and methylerythritol 4–phosphate pathways are involved in terpene synthesis (Verma & Shukla, 2015)

Many terpenoids possess various biological activities. E.g. monoterpenes as geraniol, citronellol, citral, cuminaldehyde, carvacrol possess anti–anisakidosis larvae activity (Hierro et al., 2004); linalool, carvacrol, geraniol and terpinen–4–ol have shown activity against *Leishmania infantum promastigotes* (Morales et al., 2009). Limonene showed antitumoral, antibiotic, antiprotozoal and antileishmania activity (Arruda et al., 2009; Del Toro–Arreola et al., 2005); Bound et al. (2007) reported that glucosides of carvacrol, thymol, and perillyl alcohol possess anti–fungal activity against *Aspergillus flavus*, *Aspergillus ochraceus*, *Fusarium oxysporum*, *Saccharomyces cerevisiae* and *Candida albicans*. Fernandes et al., (2013) reported that diterpene jatrophone and sesquiterpene cyperenoic acid extracted with hexane from *Jatropha ribifolia* roots, demonstrated anti–tumor/antiproliferative effects against ten human cancer cell lines: glioma, melanoma, breast cancer, adriamycin–resistant ovarian cancer, kidney, non–small lung cancer, ovarian cancer, prostate cancer, colon cancer, leukemia and normal green monkey kidney cells. Also, sesquiterpene nerolidol possessed antifungal (Oliva et al., 2003) and antileishmania effect (Arruda et al., 2005).

2.2.2. Phenolic compounds

The second major group of PSMs belongs to phenolic compounds, which are responsible for many physiological roles in plants: (i) growth, (ii) development (iii) reproduction (iv) defence against biotic (viruses, bacteria, fungi, nematodes, insects and arachnids) and abiotic stress (light, temperature, drought, pollution) (Harborne & Williams, 2000). Phenolics are the compounds with phenyl ring bearing one or more hydroxyl group (Achakzai et al., 2009).

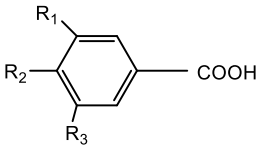
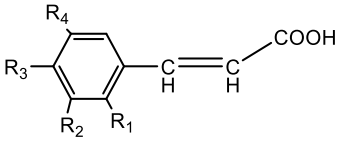
The phenolic compounds have been divided into different groups, depending on the number of phenol rings and the structural elements that bind these rings to one another (**Fig. 2.5.**). They can be classified by the number and arrangement of their carbon and are commonly found conjugated to sugars and organic acids (Crozier et al., 2009). Simple phenolic compounds are known as phenylpropanoids such as trans–cinnamic acids, benzoic acid and its derivatives.

2.2.2.1. Phenolic acids

Phenolic acids (PA) are usually present in the bound soluble form conjugated with sugars or organic acids and are typically components of complex structures such as lignins and hydrolyzable tannins (**Table 2.1)**. PAs play diverse functions in plants, including (i) nutrient uptake, (ii) protein synthesis, (iii) enzyme activity, (iv)

photosynthesis, (v) structural components (Wu et al., 1999, 2000). Many of the health protective effects of PA have been ascribed, including antibacterial, anti–inflammatory, antiviral, radical scvanenging, anticarcinogenic (Duthie et al., 2000, Cheynier, 2005; Crozier et al., 2009).

Table 2.1. The chemical structures of phenolic acids

Class	Structure	Trivial name
Benzoic acids		Gallic a. $R_1=R_2=R_3=OH$ Protocatechuic a. $R_1=H$, $R_2=R_3=OH$ Vanillic a. $R_1=H$, $R_2=OH$, $R_3=OCH_3$ Syringic a. $R_2=OH$, $R_1=R_3=OCH_3$
Cinnamic acids		Ferulic a. $R_1=R_2=H$, $R_3=OH$, $R_4=OCH_3$ *ρ*–Coumaric a. $R_1=R_2=R_4=H$, $R_3=OH$ Caffeic a. $R_1=R_2=H$, $R_3=R_4=OH$ Sinapic a. $R_1=H$, $R_3=OH$, $R_2=R_4=OCH_3$

PAs, commonly found in large families of MAP as Asteraceae, Rosaceae and Lamiaceae, are represented by 4–hydroxybenzoic, vanillic, chlorogenic, syringic, *o*–coumaric, *p*–coumaric, ferulic, hesperetic, *p*–anisic, salicylic, cinnamic, and methoxycinnamic acids (Sytar et al., 2016). Generally, hydroxycinnamic acids as caffeic, *p*–coumaric and ferulic are found in foods as simple esters with glucose and quinic acid. The most common hydroxycinnamic acid ester is chlorogenic acid. Hydroxycinnamic acid derivatives were found to possess antioxidative effects (Mattila & Hellstrom, 2007) and were applied in the prevention of cancer and cardiovascular diseases (Luthria and Mukhopadhyay, 2006; Ness and Powles, 1997). E. g. one of the most common naturally found cinnamic acid is caffeic acid, which was reported to selectively block the biosynthesis of leukotrienes, the components involved in immunoregulation diseases asthma and allergic reactions (Koshihara et al., 1984). Other studies have showed that cafeic acid and its derivatives possess antitumor activity (Olthof et al, 2001; Robbins, 2003). PA were found to be responsible for food sensory qualities, colour, antioxidative and nutritional properties. Additionally, the content of phenolic acids have been connected with (i) influence on fruit ripeness (ii) prevention of enzymatic oxidation (browning) and

(iii) their roles as food preservatives (Tomas–Barbera and Espin, 2001; Shahidi and Nacsk, 1998; Fernandez–Zurbano et al., 1998).

2.2.2.2. Flavonoids

Flavonoids is the most common group of phenolic compounds that naturally occur in plant-based foods and drinks including tea, coffee, wine, juices; they are also found in various fruit, vegetables, grains and herbs. It is usually detected in epidermis of plant leaves and skin of fruits. The sceleton of falavonoid (**Fig 2.5**) consist of two aromatic rings connected by a three-carbon bridge C6–C3–C6 (Crozier et al., 2009). The main subclasses of dietary flavonoids are: flavonols, flavones, flavan–3–ols, anthocyanidins, flavanones and isoflavones. Various funcions are asigned toflavonoids in the plants: (i) UV photoprotection, (ii) reproduction, (iii) internal regulation of plant cell physiology and (iv) signaling. Some flavonoids provide stress protection; for example, acting as scavengers of free radicals such as reactive oxygen species (ROS), as well as chelating metals that generate ROS via the Fenton reaction (Williams et al., 2004). Various flavanoids possess antiviral, antiplatelet, anti–inflammatory, antimicrobial, antioxidative, hepatic steatosis and hypolipidemic effects; they also serve as possible neuroprotective agents in progressive neurodegenerative disorders such as Parkinson's and Alzheimer's diseases and are related to reducing the risk of heart deseases. (Bansode et al., 2014; Ali & Dixit, 2012; Weinreb et al., 2004 Agati et al., 2012, Del Rio et al., 2010, Nijveldt et al., 2001).

Flavonols are abundant throughout the plant kingdom except for fungi and algae. The most common flavonols, kaempferol, quercetin, isorhametin, and myricetin (**Fig 2.5**) are typically found as glycosides with conjugation occurring at the 5, 7, 3‘,4‘, and 5‘positions. (Del Rio et al., 2013). Fistein and quercetin found in high concentrations in grapes, onion, tea, cucumber, apple possess anti–prostate cancer properties (Khan et al., 2008; Hsieh &Wu, 2009; Senhilkumar et al., 2011).

Flavones, such as apigenin, luteolin, wogonin, and baicalein, a sub–class of flovanoids is the most abudant in artichoke heads, kumquats, parsley, and celery. Apigenin showed anti–cancer (Mak et al., 2006) and anti–inflammatory effects (Nicholas et al., 2007). The other dietary flavone – luteolin possesses antioxidant and antitumor activities (Kasala et al. 2016), as well as antiviral effect against Japanese encephalitis virus (Fan et al., 2016) and antidipogenic and proosteogenic effects (Kwon et al., 2016).

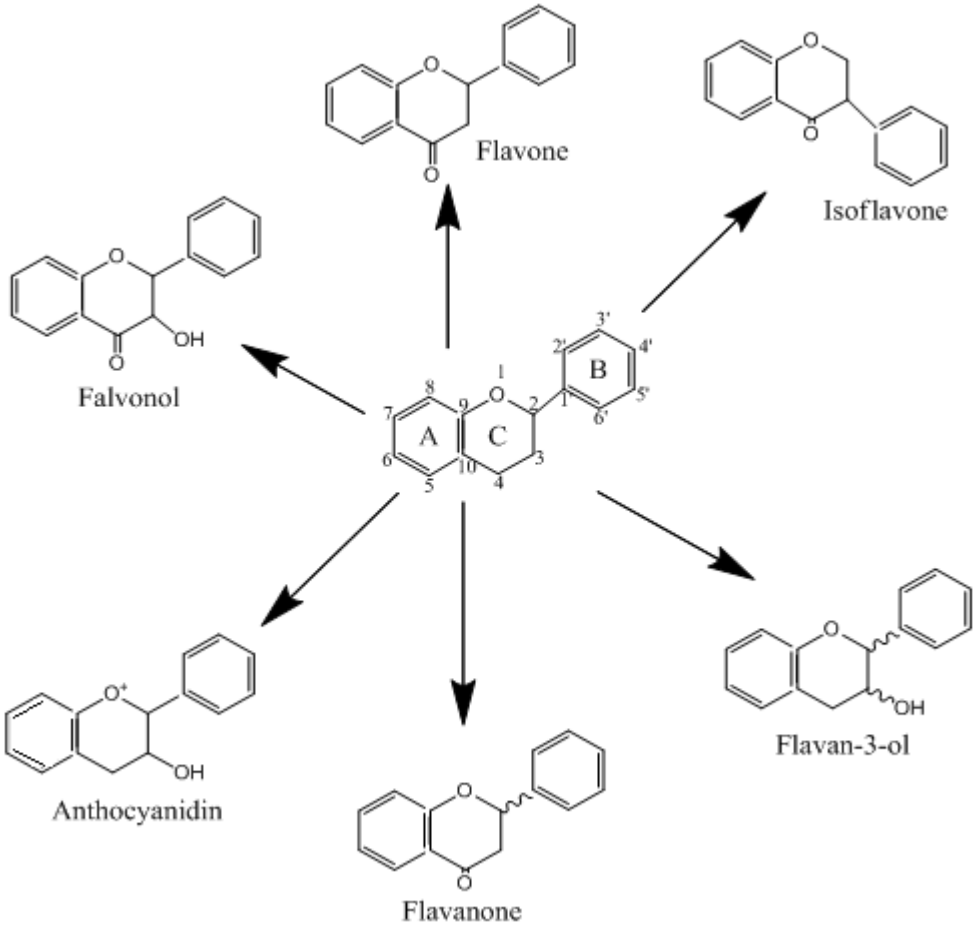


Fig. 2.5. Basic structures of flavonoids and its sub–classes (Crozier et al., 2009).

Isoflavones, genistin and daidzin usually are abundant in soy plants and beans. Isoflavones have the B–ring attached at C–3 rather than at the C–2 position (**Fig. 2.5**) Gao et al. (2003) reported that daidzin has been shown to reduce alcohol consumption. Because of their structural similarity to estrogen, isoflavones are classified as phytoestrogens, as are the non– flavonoid lignans, which are a diverse group of compounds that occur in high concentrations principally in cereal grains (Del Rio et al., 2013). Flavanones occur as hydroxyl, glycosylated, and O–methylated derivatives. They are present in especially high amounts in flavedo of citrus fruits. The most common flavanone glycoside is hesperetin–7–O–rutinoside (hesperidin). Devi et al. (2015) reviewed that hesperidin in addition to citrus fruits has been detected in different species of Fabaceae (such as *Cyclopia* species, *etc*.), Betulaceae (such as *Carpinus* species, *etc.*), Lamiaceae (such as *Mentha* species, *etc*.) and Papilionaceae (such as *Pterocarpus* species, *etc*.) families. It showed anticancer (Devi et al., 2015) and possessed protective effects against microbes and toxins (Iranshahi et al., 2015). Omar et al., (2016) revealed that

hesperidin, a naturally occurring flavanone in honeybush (*Cyclopia maculata*), showed protective effects against cisplatin–induced acute hepatotoxicity in rats (Preez et al., 2016).

Pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin are the most common anthocyanidins (aglycones of anthocyanins), which provide the red, blue and purple pigmentation to vegetables and fruits (Longo & Vasapollo 2006; Ekici, 2011). They are ussually present in conjugates with sugars and organic acids (Del Rio et al., 2013). Antioxidant, cardio–protective, anti–inflammatory and anti–carcinogenic effects have been observed from the dietary intake of food rich in anthocyanins in both human and other mammalian models (Villasante et al., 2016).

Flavan–3–ols are the most complex subclass of flavonoids, ranging from the simple monomers to the oligomeric and polymeric proanthocyanidins, which are also known as condensed tannins. Catechin, a flavanol, and its derivatives found in green tea, is conected with preventive and therapeutical potential against periodantal desease (Del Rio et al., 2013).

In food industry flavanoids are used as food pigments, flavorants, and preservative agents due to their color and flavour, antioxidative and anti–microbial properties.

2.2.2.3. Biosynthesis of phenolic compounds

The phenolic compounds in higher plants are basically formed by shikimic acid pathway (**Fig. 2.6** adapted Cheynier et al., 2013). Shikimic acid is the intermediate in this pathway. First, phosphoenolpyruvate (PEP) and erythrose–4–phosphate (E4P) derived from metabolism of glucose are converted into different aromatic amino acids in which the most frequent intermediate is phenylalanine (Taiz and Zeiger, 2006). Trans cinamic acid is formed by deamination of pheylalanine by enzyme phenylalanine ammonialyase (PAL). PAL plays a pivotal role in phenolic synthesis. Huge number of reports maintain the interface between PAL activity and increases in phenolic compounds in response to different stimuli (Boudet, 2007). Cinnamic acid is modified to *p*–coumaric acid by cinnamate–4–hydroxylase (C4H) and o–methyltransferase. The enzyme 4–coumarate CoA ligase (4CL) catalyzes the formation of Coumaroyl CoA and these activated intermediates are used in the biosynthesis of monolignols, lignans and lignin.

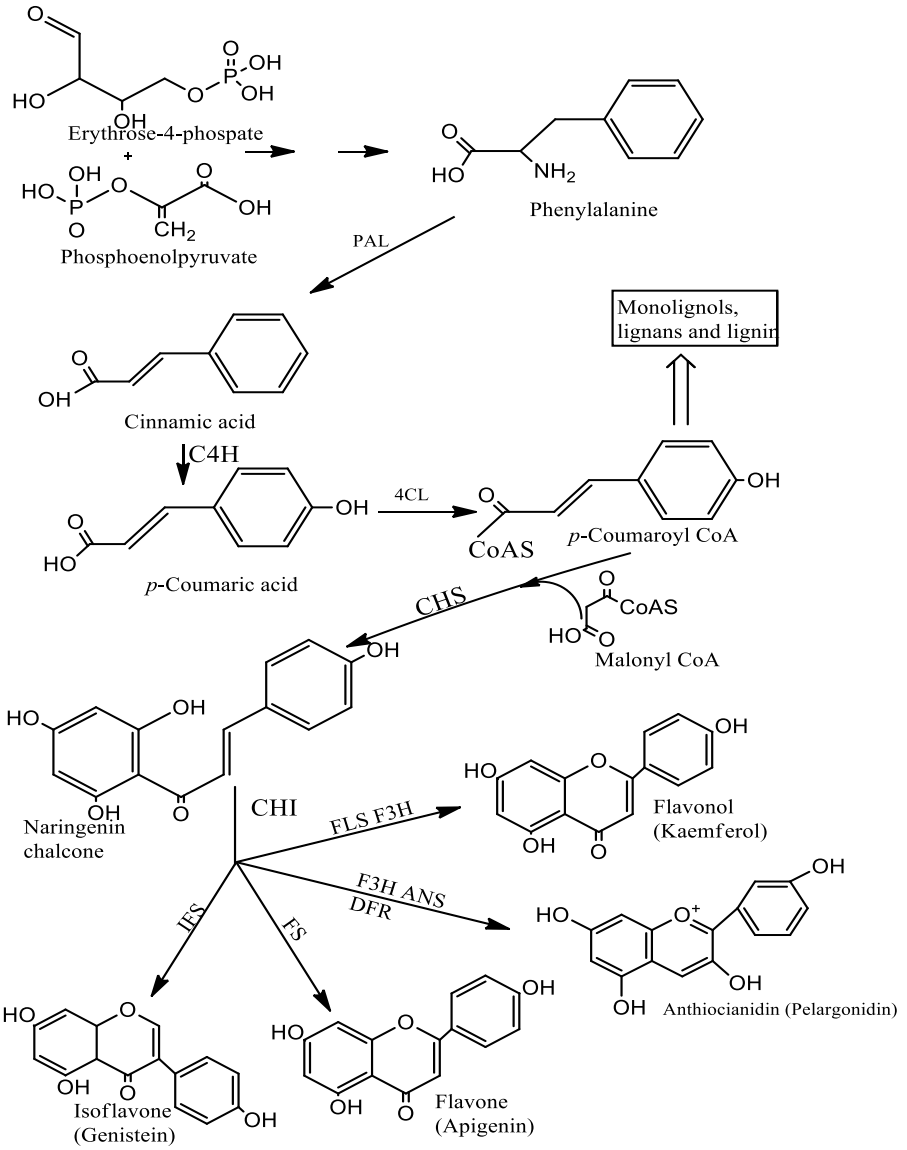


Fig. 2.6. Schematic of the major branch pathways of (poly)phenol biosynthesis. PAL, phenylalanine ammonialyase; C4H, cinnamate–4–hydroxylase; 4CL, 4–coumaroyl:CoA–ligase; HCT, hydroxycinnamoyl transferase; C3H, p–coumarate–3–hydroxylase; CHS, chalcone synthase; CHI, chalcone isomerase; ANS, anthocyanidin synthase; DFR, dihydroflavonol reductase; FS, flavone synthase; FLS, flavonol synthase; F3H, flavanone 3–hydroxylase; IFS, isoflavone synthase; ANR, anthocyanidin reductase; LAR, leucoanthocyanidin reductase (addapted Cheynier et al., 2013)

Flavonoids synthesis starts with the condensation of *p*–Coumaroyl coumaroyl–CoA and three molecules of malonyl CoA catalyzed by chalcone synthase (CHS). Naringenin chalcone is further converted to other flavonoid subgroups, including

flavone, isoflavone, flavonol and, anthocyanidin by chalcone isomerase (CHI), flavanone 3–hydroxylase (F3H), isoflavone synthase (ISF), flavone synthase (FS), flavonol synthase (FLS), dihydroflavonol reductase (DFR) and, anthocyanin synthase (ANS) (Sakihama et al., 2002).

2.2.2.4. Antioxidative activity measurements *in vitro*

What is oxidative stress (OS)? It is one of the most commonly raised questions in human physiology. Shortly, OS is defined as the imbalance between the reactive oxygen species (ROS) and antioxidant levels in favor of prooxidants in cells and tissues (Kalyanaraman, 2013). The result of OS is modifications of lipids, proteins and DNA. Antioxidant is a molecule inhibiting the formation of prooxidants and oxidation. Phenolic compounds behave as antioxidants, due to the reactivity of the phenol moiety (hydroxyl substituent on the aromatic ring). Phenolic compounds can easily donate hydrogen from hydroxyl groups positioned along the aromatic ring to terminate free radical oxidation of lipids or other biomolecules. The aromatic phenolic ring can stabilize and delocalize the unpaired electron within its aromatic ring (**Fig. 2.7**) (Bhanja Dey et al., 2016). The key point of antioxidative activity(AA) is related with structure of phenolic compound. E.g. AA of phenolic acids depends on the numbers and positions of the hydroxyl groups in relation to the carboxyl functional group. The AA of flovanoids is more complicated, it depends on the degree of hydroxilation, the OH groups and double bonds position and substitutes of hydroxyl groups (Balasundram et al., 2006).

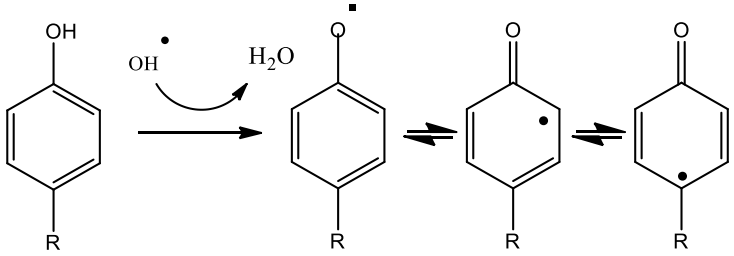


Fig.2.7. Free radical scavenging antioxidant mechanism of phenolic compound

There are many *in vitro* radical scavenging assays, whereof majority of them are based on single electron transfer (SET) and hydrogen atom transfer (HAT) reactions (**Table 2.2**). Huang et al. (2005) concluded that Oxygen Radical Absorbance Capacity (ORAC), Total Phenolic Content (TPC) measured with Folin–Ciocalteu reagent and one of the SET/HAT assays should be recommended for the representative evaluation of antioxidant properties.

ORAC is standardized AOC method utilizing HAT reaction mechanism. It measures antioxidant inhibition of peroxyl radical induced oxidations and thus reflects classical radical chain breaking antioxidant activity by H atom transfer (Ou et al., 2001).

$ROO^{\bullet} + AH/PheOH \rightarrow ROOH + A^{\bullet}/Phe–O^{\bullet}$, where AH is any H donor, PheOH = phenol or polyphenol.

Peroxyl radical is the most prevalent free radical in human biology, therefore ORAC is better related to *in vivo* conditions (Prior, 2015). This method was widely used to test antioxidative properties of various herbal extracts (Baranauskienė et al., 2014; Lucini et al., 2015) and food samples, including orange juice, milk (Zulueta et al., 2009), vegetables (Kameya, 2014), fruits (Bravo et al., 2016) berries (Kraujalytė et al., 2013, 2015)

Table 2.2. *In vitro* antioxidant capacity (AOC) methods (Prior et al., 2015)

Antioxidant capacity methods (AOC) based on hydrogen atom transfer (HAT)	➢ ORAC (Oxygen Radical Absorbance Capacity) ➢ TRAP (Total Radical Trapping Antioxidant Parameter) ➢ IOU (Inhibited Oxygen Uptake) ➢ Inhibition linoleic acid oxidation ➢ Inhibition of LDL oxidation ➢ PCL (Photochemiluminescence Assay)
Antioxidant capacity methods based on single electron transfer (SET)	• FRAP (Ferric Reducing Antioxidant Power) • CUPRAC, (Copper (II) Reducing Power) • TEAC (Trolox Equivalent antioxidant capacity) or Other ABTS Assays • DPPH (2,2–Diphenyl–1–picrylhydrazyl) Assay • Total Phenolic Content (TPC) using Folin–Ciocalteu reagent
Other assays	✓ TOSC (Total Oxidant Scavenging Capacity) ✓ Inhibition of Briggs–Rauscher oscillation reaction ✓ Chemiluminescence ✓ Electrochemiliminescence

The $ABTS^{\bullet+}$ radical cation used in TEAC assays is not found in mammalian biology and thus represents a "nonphysiological" radical source (Prior et al., 2005). TEAC measures antioxidant capacity as the ability of test antioxidants (AH) to reduce $ABTS^{\bullet+}$ colour by (a) intercepting initial oxidation and preventing $ABTS^{\bullet+}$ production, or (b) reacting directly with the $ABTS^{\bullet+}$ (Schaich et al., 2015). In general, ABTS and FC (total phenolic content using Folin-Ciocalteu reagent) assays represents single electron transfer (SET) methods:

$ROO^{\bullet} + AH/\ PheOH \rightarrow ROO^{-} + AH^{+}/\ PheOH^{+} \longrightarrow A^{\bullet}/Phe–O^{\bullet} + H_3O^{+}$

$ROO^{-} + H_3O^{+} \rightarrow ROOH + H_2O$, where AH is any H donor, PheOH = phenol or polyphenol. The advantage of the ABTS test is its relative simplicity that allows its application for routine determinations in any laboratory (Roginski and Lissi, 2005). FC assay was not supposed to characterize AOA, however currently this method seems to be one of the best for rough estimation of AOA of food samples if the sample tested does not contain proteins in significant amounts. (Roginski and Lissi, 2005).

Measurement of antioxidant capacity described above requires an extraction step and/or hydrolysis of bound antioxidant compounds from the insoluble matrix (Kocadağlı & Gökmen). However, most of the foods are composed of the mixture of soluble in different solvents and completely insoluble bioactive compounds, which can be in free form and bound with macromolecules (Gökmen et al., 2009) The direct QUENCHER procedure, described as Quick, Easy, New, Cheap and Reproducible, does not requires the extraction step and can measure antioxidant activity directly in the solid matrix. QUENCHER procedure was applied to measure antioxidative activity in solid matrices, including coffee beans (Kocadağlı & Gökmen), cereals (Tufan et al., 2013, Rufian–Henares & Delgado–Andrade, 2009), nuts, seeds (Acar et al., 2009) various herbs (Šiulniūtė et al., 2016, Kemzūraitė et al., 2014).

2.2.3. Nitrogen containing compounds

The third important category of secondary metabolites includes nitrogen–containing compounds such as alkaloids, glucosinolates and cyanogenic glycosides. Alkaloids are low molecular weight compounds, synthesized from few amino acids like lysine, tyrosine and tryptophan (Taiz & Zeiger, 2006). They may be responsible for taste and odour, therefore are linked with the protective role in plants against pests and pathogens (Taiz and Zeiger, 2006). Some alkaloids as vinblastine and amaline showed antibacterial activity and possible beneficial effects against cancer and heart diseases (Lee et al, 2016, Conte et al., 2014). Other alkaloids containing phenol group – avenanthramides in oats extracts showed high antioxidative effect *in vitro* and *in vivo* (Chen et al., 2007).

2.2.4. Medicinal and aromatic plants in food and medicine

Spices and herbs have been used as sources of phytochemicals, pharmaceuticals, nutraceutical constituents, herbal remedies, food supplements, perfumes and cosmetics, and food flavourings, colouring, preservating agents for more than 2000 years (Embuscado, 2015; Kumar et al., 2016). The commercialization of medicinal plants has considerably increased due to their large-scale use in above mentioned sectors. According to WHO estimates, the present demand for medicinal plants is worth $14 billion annually, and by the year 2050, it will be $5 trillion. A lot of MAPs is used in the production of salads, desserts, ice cream also for food decoration, it also works as food preservatives and is used in cosmetic industry and pharmacology (**Table 2.3**). The plants from several families, especially Lamiaceae (rosemary, sage, oregano, marjoram, basil, thyme, mints, balm), Apiaceae (cumin, fennel, caraway), and Zingiberaceae (turmeric, ginger) are worldwide known and commonly used medicinal plants (Škrovankova et al., 2012).

Thymus serpyllum and *T. vulgaris*, belonging to Lamiaceae family are traditionally used worldwide to treat respiratory tract infections. The essential oil (EO) production remains one of the major reasons for thyme cultivation. Thyme EO was reported to demonstrate antimicrobial, anti–inflammatory, antinociceptive and spasmolytic activities (Prosen & Pendry, 2016, Gavaric et al., 2015). Boskovic et al.

(2015) reported that *T. vulgaris* and *Origanum vulgare* EOs possessed antimicrobial activities in meat against *Salmonella enteritidis, Salmonella Thyphimurium*, *Staphylococcus aureus*, methicillin resistant *Staphylococcus aureus*, *Escherichia coli* and *Bacillus cereus*. The antifungal activity of carvacrol and thymol (the main EO components of some Lamiaceae plants) as well as their synergistic potential with antibiotics, has been described previously (Jesus et al., 2015). The small seeds of cumin (*Cuminum cyminum*) are a popular spice in the world from Latin America to Northern Africa and all over the Asia and used as a flavouring agent in many products such as cheese, pickle, soup, bean dishes or liqueurs. Essential oils of the seeds are also used as a flavour in aromatherapy. They possess anti–diabetic, immunologic, anti–epileptic, anti–tumour and antimicrobial activities. (Gohari & Saeidnia, 2011). Turmeric *Curcuma longa* (Zingiberaceae) is edible plant with pharmacological activities. E.g. its powder showed effects in treating irritable bowel syndrome, peptic ulcer, and inflammatory bowel disease (Monton et al., 2016). Saffron (*Crocus sativus*) is the most expensive spice in the world, which is traditionally used as a colouring or flavouring agent. Bayram et al., (2015) concluded that saffron, rose (*Rosa damascena*) lavender (*Lavandula angustifolia*), verbena (*Verbena officinalis)* and some others are the most popular edible flowers in Turkey. Despite their taste, attractive colour and beauty they posses antiradical capacity, anticancer functions, works as cardiovascular health promoters, possess antimicrobial, antiseptic, anti–inflammatory, antidepressant, antiviral and antibacterial activities (Baba et al., 2015; Melnyk et al., 2010; Mahboubi, 2016; Nikolova et al., 2016; Encelada et al., 2015).

Table 2.3. Ingredients from commonly used MAP for pioneering industrial application

Industrial segment	Family	Activities	Target product	References
Cosmetics	Lamiaceae (*Rosmarinus spp.*; *R. officianalis, O. majorana*)	Antimicrobial, anti–inflammatory	Anti–acne skin agent, skin aging skin lightening	Lee et al., 2011; Baylac&Racine, 2004; Boissy et. Al., 2005)
	Rosaceae (*Rosa damascena*),	Antioxidant, antifungal, antibacterial	Perfumes, essential oils	Mahboubi, 2016; Nasery et al., 2016
Food	Lamiaceae (*Lavandula angustifolia ajugaiva; M. vulgare, M pulegium, M. myrtifolia, C. origanifolia*)	Antimicrobial, antioxidant, anticollagenase, antioxidant, stabilization against thermo oxidation, antibacterial	Skin aging, food preservatives and additives, food decoration, edible flower,	Bayram et al., 2015; Thring et al., 2009
	Verbaceae (*Verbena officinalis*)	Antioxidant	Food decoration, edible flower	Bayram et al., 2015
	Apiaceae *(Ridolfia segetum, Ferula heuffelii)*	Antioxidant, anti–inflammatory, antimicrobial atispasmotic	Food preservative	Cabral et al., 2015, Pavlovic et al., 2012
Pharmacological	Zingiberaceae *(Renealmia alpinia)*	antiedematous, antihemorrhagic, antimalarial, leishmanicidal activities, antiproliferative effect on tumor cells	Antidote against snakebites	Gómez–Betancur& Benjumea, 2014
	Lamiaceae *(T. Villosus subsp. Lusitanicus, thymus spp., T. Sipthorpii and S. aintabensis)*	Antimicrobial against *Candida* and *Aspergillus*, antimicrobial against multidrugs–resistant bacteria, antibacterial activity against *M. tuberculosis*	Anti–dermatomycosis drug, antibiotics, antituberculosis drug	Pinto et al., 2013, Nabavi et al., 2015, Askun et al., 2013

2.3. The improved utilization of plant biomass

The increment of human population following to the new challenges in basic requirements (housing, mobility, energy, food and water), the economical drivers such as global warming, insecurities in energy supply and agricultural policies are

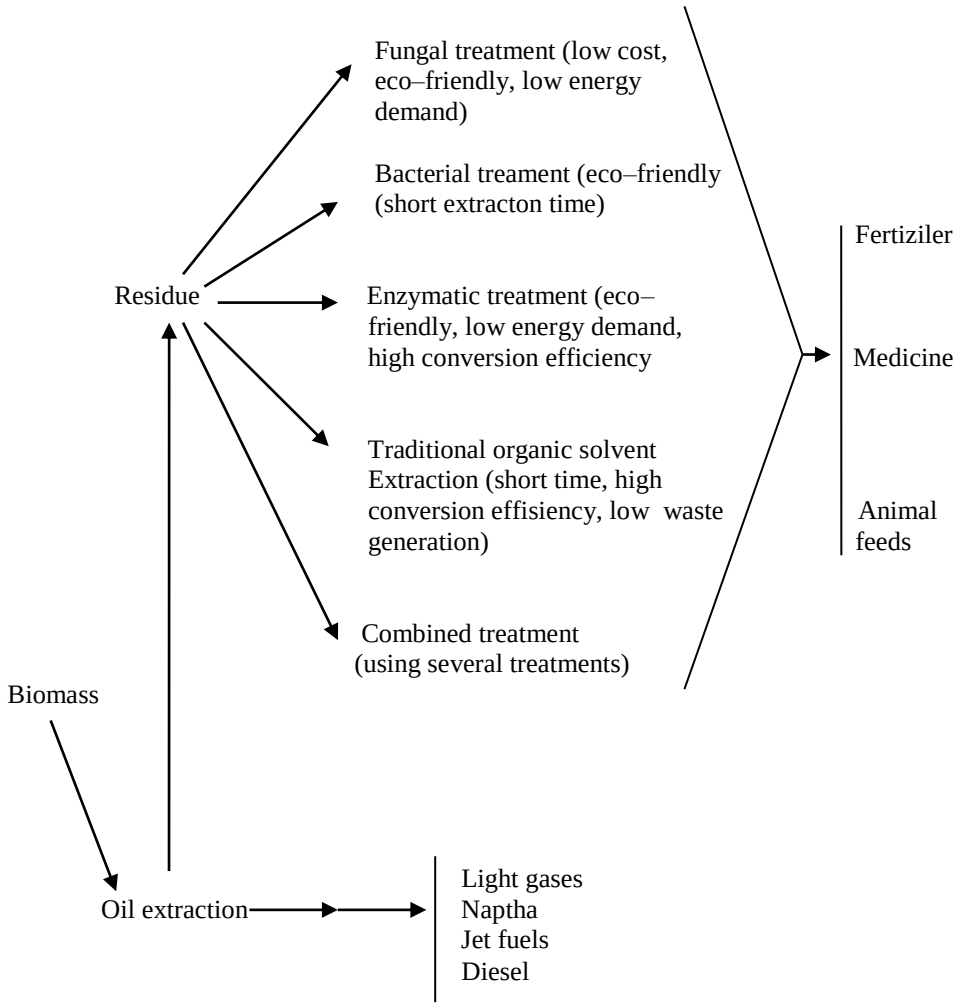


Fig. 2.8. Biorefinery of by–products

further expected to improve their operations in a sustainable biorefinery manner. Biorefineries are promising integrated process facilities generating biomass into multiple products: (i) food, (ii) feed, (iii) materials, (iv) chemicals and (v) energy with zero waste generation (**Fig 2.8**). Biorefinery must be able to use a wide range of biomass, including forestry, agriculture, aquaculture and residues from industry and

households. It is not a new concept as traditional biomass processing can be partially considered as biorefinery (Abraham et al., 2016). Nowadays the limitation of organic solvents in food industry, concerning food products safety, is very important and widely discussed. Hence, the new and combined extraction techniques (such as supercritical fluid extraction and pressurized liquid extraction,), avoids the using of huge amounts of organic solvents and enlarge opportunities for recovery and application of various antioxidants and other phytochemicals for foods, nutraceuticals, cosmetics, medicinal and other purposes.

2.3.1. Supercritical fluid extraction

In recent years, the demand of natural bioactive compounds, such as polyphenols, carotenoids, peptides, sterols or poly unsaturated fatty acids (PUFAs), has increased particularly due to increasing market of functional foods and nutraceuticals. The limitation of organic solvents, concerning food products safety is very important and widely discussed question. Hence, novel green extraction techniques (such as supercritical fluid extraction, (SFE)), which are more environmentally safe techniques than the convectional extraction methods are of interest (Fathordoobady et al., 2016).

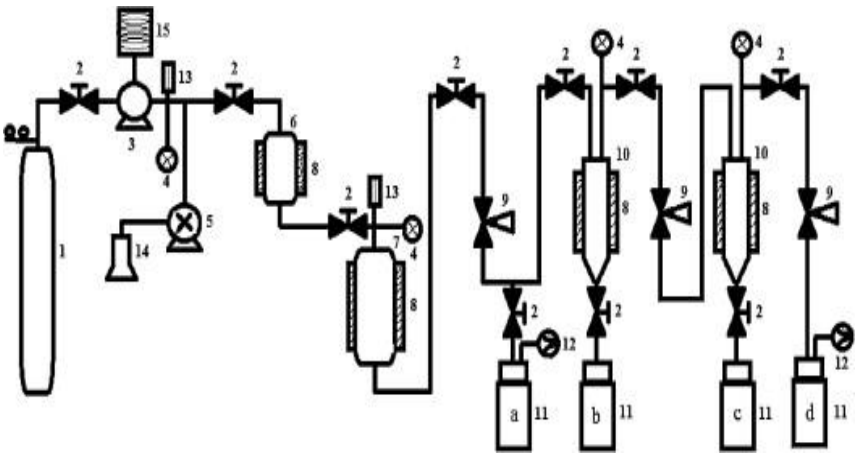


Fig. 2.9. Schematic diagram of SCE–CO_2 equipment: 1 – CO_2 cylinder; 2 – on–off valve; 3 – CO_2 pump; 4 – pressure gauge; 5 – cosolvent pump; 6 – CO_2 vessel; 7 –extraction vessel; 8 – heat jacket; 9 – manual back pressure regulator; 10 – separator; 11 – collection vials (a, b, c, d); 12 – gas flow metre; 13 – safety valve; 14 – cosolvent; 15 – CO_2 pump chiller. (Adapted from Kraujalis & Venskutonis et al., (2013).

SFE is a green extraction technology that has been broadly applied for the recovery of valuable compounds from different materials, both at laboratory and industrial levels (**Fig 2.9**). SFE is based on the use of solvents above their critical pressures and temperatures. At those conditions, supercritical fluid possess particular physico–chemical characteristics between gases and liquids, generally

acquiring higher density than a gas but maintaining similar viscosities and intermediate diffusivities (Herrero et al., 2015). Carbon dioxide is the most widely used supercritical fluid for the recovery of bioactive and valuable compounds from natural matrices. CO_2 presents several advantages: (i) mild critical conditions, (ii) non–toxic, (iii) non–flammable, (iv) non–explosive, (v) easily available, (vi) cheap, and (vii) easily eliminated from extracts (Herrero et al., 2015). A disadvantage of CO_2 is its low polarity. To overcome this issue co-solvents (solvents with higher polarity than CO_2, also called modifiers) are employed during extraction at small proportions (typically, 1–10%). A lot of factors are involved in SFE process, including (i) supercritical solvent, (ii) nature of modifier, (iii) proportions of modifier, (iv) temperature, (v) pressure, also the parameters of the sample (i) particle size, (ii) moisture, (iii) dispersant agent, (iv) amount, (v) supercritical fluid flow rate, (vi) extraction time and (vii) fractionation possibilities. SFE is widely used for bioactive compounds extraction from plants, macro and microalgae, food–related by–products (Herrero et al., 2015). E.g. carotenoids, which are the main pigments in many plants, are known for their bioactivities including antioxidant, anti–proliferative, anti–inflammatory, provitamin A activity, and even protection of macular degeneration (Fernandez–García et al., 2012), have been recovered from different plant matrices using SFE (Durante et al., 2014; Prado et al., 2014). Residues from processing of berries (Grunovaitė et al., 2016, Kryževičiūtė et al., 2015; Paes, et al., 2014), filter cake (Prado et al., 2011) pomelo peels (He et al., 2012), have been explored for extraction of different kinds of compounds by SFE.

The main advantages of SFE techniques are as follows: (i) low extraction temperature, (ii) continuous flow of fresh fluid, (iii) faster mass transfer and (iv) high selectivity, (v) capability of handling the salvation power of solvent by altering pressure and/or temperature (Fathordoobady et al., 2016).

2.3.2. Pressurized liquid extraction

The general term for this process (PLE) involves the application of an extraction technique that has also been referred in the literature as pressurized fluid extraction (PFE), pressurized hot–solvent extraction (PHSE) or accelerated solvent extraction (ASE).

In any case, this technique is based on the use of pressurized solvents at high temperatures (however, always below their critical points), under conditions in which the solvents are maintained in the liquid state during the extraction process. When the extraction solvent is water, this technique is also called subcritical water extraction (SWE), superheated water extraction (SHWE) or pressurized hot–water extraction (PHWE) (Herrero et al., 2015). Comparing PLE with other traditional extraction processes (as Soxhlet extraction) it (i) requires smaller solvent quantities, (ii) is much faster and (iii) fully automated. In general, the instrumentation comprises a high–pressure pump to push the solvent into an extraction cell, which is maintained in an oven to control the extraction temperature, with different valves and restrictors to control the extraction pressure (**Fig. 2.10**). The system may include other additional parts, such as solvent–controller valves, nitrogen–purging lines,

extraction cells, and vial trays for automation. Automation helps to reduce extraction–to–extraction variation and to increase the reproducibility (Herrero et al., 2015).

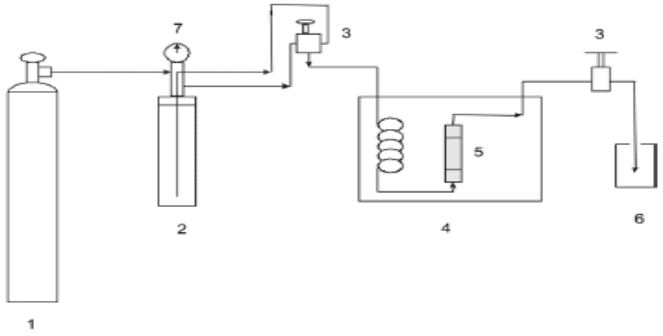


Fig. 2.10. Schematic diagram of the pressurized liquid extraction (PLE) system. 1–nitrogen tank, 2–pressure vessel, 3–valves, 4–oven, 5–extraction cell with filters, 6–collector flask, 7–monometer

PLE has been widely employed for the extraction of bioactive compounds from plants, micro and macro algae and food–related by–products. E.g. phenolic acids, anthocyanins were extracted from potato peels (Luthria, 2012; Casas–Cardoso et al., 2013), flavonols from apple by–products (Plaza et al., 2013), antibacterials from ginseng stems and leaves (Lee et al., 2013), tannins from grape pomace (Vergara–Salinas et al., 2013), carotenoids and antimicrobials from microalgae (Herrero et al., 2006; Santoyo et al., 2009), ω–3 fatty acids from wastewater (Mulbry et al., 2009).

2.4. Summary of literature survey

The literature survey shows that plant biodiversity is very high; however due to increasing consumer's demand it is important to look for the new bioactive components and their sources, to employ new environment friendly extraction techniques. Previously performed studies applied conventional solvent extraction methods mainly using highly toxic solvent methanol and were performed mainly for the analytical purposes, while more comprehensive biorefining schemes of selected plants into valuable fractions, which could be used for up scaling to pilot and industrial production, have not been investigated until now. Moreover, to the best of our knowledge, high pressure methods such as supercritical fluid extraction with carbon dioxide (SFE–CO_2) and pressurized liquid extraction (PLE) have not been applied for the isolation and fractionation of antioxidants and phytochemicals from selected plants previously. So far as these methods possess several advantages, systematic studies of their application for bergenia, buckwheat and goldenrod different anatomical parts in obtaining high value nutritional fractions with antioxidant and other bioactivities may be of great interest in the development of natural functional ingredients suitable for various applications.

III. MATERIALS AND METHODS

3.1. Chemicals and plant material

Roots and leaves of *B. crassifolia* (further referred as BR and BL), buckwheat flowering parts (BWF) and European goldenrod leaves (GRL) were collected in September 2012 (BR and BL) and 2013 (BWF and GRL) in organic herb farm located in Dzūkija National Park, Panara village, Varėna district, Lithuania.

The plants were dried by active ventilation using a solar collector for air heating and stored in the dark. Agricultural origin ethanol was from Stumbras (Kaunas, Lithuania), acetone, hexane and methanol from Chempur (Piekary Ślaskie, Poland). Reference compounds, citric and chlorogenic acids, isoorientin, myricitrin, quercetin, quercetin 3–D–galactoside, quercetin 3–O–α–L arabinopyranoside, quercitrin hydrate, rutin, ABTS (2,2'–azino–bis (3–ethylbenzthiazoline–6–sulphonic acid) and HPLC grade solvents used for chromatographic analyses were purchased from Sigma–Aldrich (Steinheim, Germany). Commercial refined, deodorised rapeseed oil (RO) "Tyras" without any added antioxidants was from Obelių Aliejus (Lithuania).

3.2. Sample preparation and extraction

Roots and leaves of *B. crassifolia* were collected in September 2012 meanwhile flowers of Buckwheat (*Fagopyrum Esculentum*) and Golden rod (*Solidago virgaurea* L.) leaves in September 2013 in organic herb farm located in Dzūkija National Park (Panara village, Varėna district, Lithuania. The plants were dried by active ventilation using a solar collector for air heating and stored in the dark. Dried plants samples were ground in an ultra–centrifugal mill ZM 200 (Retsch, Haan, Germany) using 0.2 mm hole size sieve according to previously published data (Kemzūraitė et al., 2014; Kraujalis et al., 2013; Kryževičiūtė et al., 2016; Šulniūtė et al., 2016). Plant examination scheme is shown in **Fig. 3.1.**

Green separation processes such as supercritical fluid extraction with carbon dioxide (SFE-CO_2) have been poorly applied for selected plants material as an alternative for the isolation and fractionation of compounds.

Supercritical fluid extraction (SFE–CO_2) was performed in a Helix extraction system (Applied Separation, Allentown, PA, USA) with 99.9% CO_2 (Gaschema, Jonava, Lithuania) from 10 g of ground material placed in a 50 cm^3 cylindrical extractor, 14 mm inner diameter and 320 mm length. Cotton wool was placed on the top and in the bottom of the extraction vessel. In all extractions CO_2 flow rate was kept constant, 2 L/min (standard conditions), static extraction time was 10 min. Extraction pressure and temperature (45 MPa, 60°C) giving high extract yields were selected based on previously published data for various botanicals (Kemzūraitė et al., 2014; Šulniūtė et al., 2016) and set automatically by PC control, while extraction time was 30, 60 and 90 min. After completing static extraction phase the flow for dynamic extraction was set by the lever according to the flow

meter reading. The CO_2 extracts were collected in glass vials and when the extraction was completed the vials were kept until constant weight to avoid CO_2 residues. The extracts were weighed and transferred to opaque bottles. In addition, SFE–CO_2 with 10% ethanol as a co–solvent was performed at 45 MPa pressure, 60 °C temperature during 60 min, which was established as sufficient for exhaustive extraction. The extracts were kept at approx. 4 °C until further analysis.

Pressurized liquid extraction (PLE) was performed in a Dionex ASE 350 system (Dionex, Sunnyvale, CA) both from the initial material and from the residues remaining after SFE–CO_2. Five g of material were mixed with diatomaceous earth (1/1) and placed in a 34 mL stainless–steel cells. The extraction was performed consecutively using the solvents of increasing polarity, namely hexane (used only for initial material for removing lipophilic substances), acetone, a mixture of ethanol/water (80/20, v/v) and water; which are further abbreviated as HX, AC, ET/W and W, respectively. Extraction time was 15 min., pressure 10.3 MPa, temperature 70 °C and 140 °C (further referred as PLE_{70} and PLE_{140}). Organic solvents were removed in a rotary vacuum evaporator at 40 °C, while the residual water was removed in a freeze dryer. The extracts after solvent evaporation were kept under nitrogen flow for 20 min and stored in dark glass bottles at −18 °C. Antioxidant properties of extracts, initial plant material and residues after extractions were evaluated using TPC, ABTS and ORAC assays.

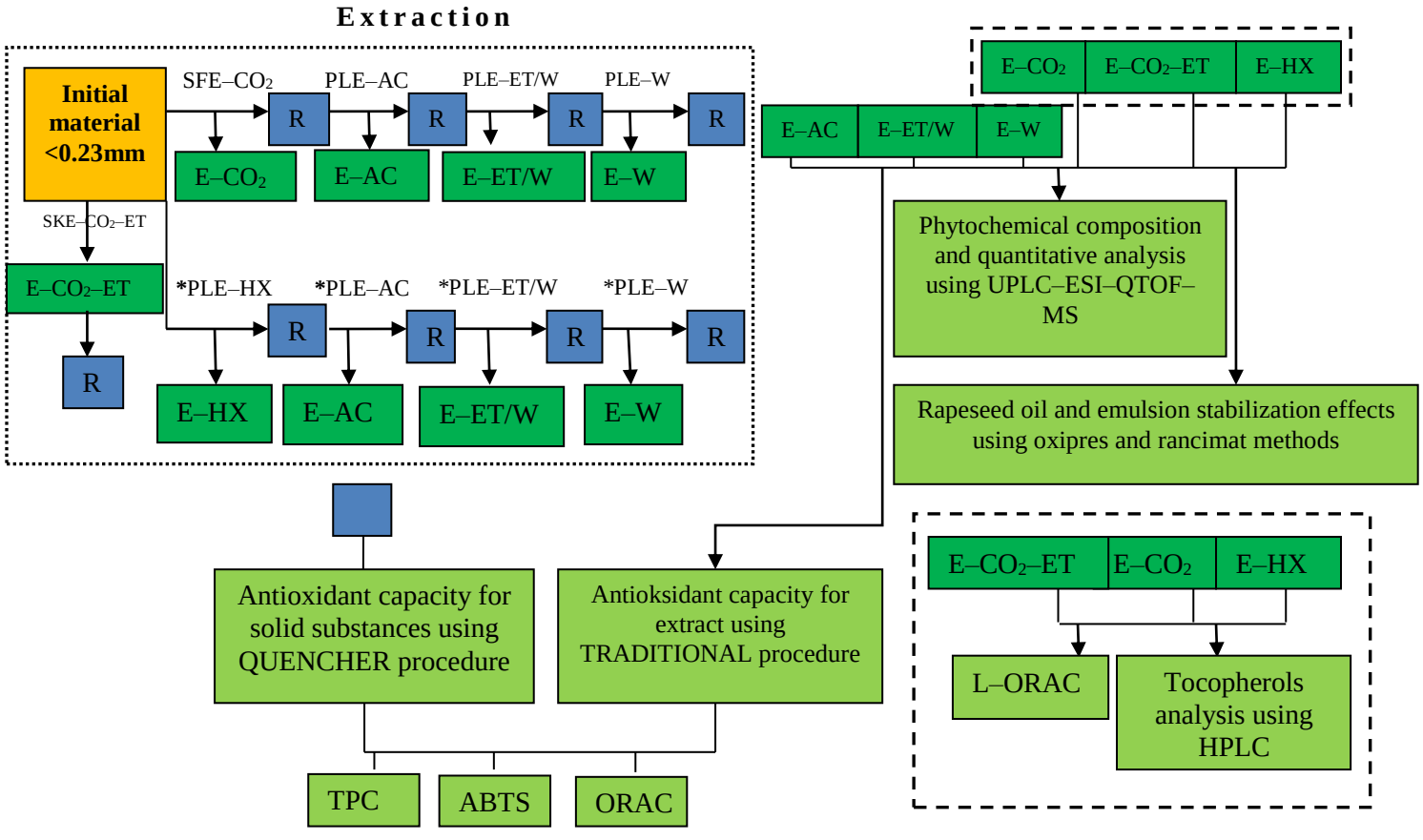
3.3. Measurements of antioxidant capacity using traditional procedure

3.3.1. ABTS•+ scavenging assay

Trolox equivalent antioxidant capacity (TEAC) was measured by using $ABTS^{\bullet+}$ scavenging assay (Re et al., 1999) with some modification. Plant extract or trolox solutions (3 μL) were reacted with 300 μL ABTS•+ solution during 90 min and the absorbance was read at 734 nm in a FLUOstar Omega reader (BMG Labtech, Offenburg, Germany). A series of trolox solutions (150–1500 μM) were used for calibration. TEAC values were calculated from the calibration curve and expressed in μmol Trolox equivalents (TE) per g dry weight of extract and plant material (μmol TE/g DWE or DWP).

3.3.2. ORAC assay

The advantage of ORAC assay is that it uses a biologically relevant radical source (Prior et al., 2003) with some modification. Fluorescence measurements (excitation at 485 nm; emission at 510 nm) were read every 66 s, in total 90 cycles. Raw data were analysed using software Mars (BMG Labtech GmbH, Offenburg, Germany). Fluorescein and AAPH solutions were prepared fresh before each measurement. Methanolic solutions (12–200 μM) of trolox were used for calibration. The ORAC assay has been also adapted to measure lipophilic CO_2 and HX extracts (Huang et sl., 2005). In this case 1 mg of extract was dissolved in 1 mL of 7% RMCD solution in acetone/water (1/1, v/v) to solubilise the antioxidants in oils. The 7% RMCD solution was used as a blank, the measurement was performed as described above.



*– pressurized liquid extraction (PLE) separately using extraction at70 °C and 140 °C;
E – extract; R – residues after extraction

Fig. 3.1. The scheme of experimental work

3.3.3. Measurement of total phenolic content (TPC)

Ten μL of appropriate dilutions of extracts or gallic acid (GA) solutions were oxidized with 190 μL Folin–Ciocalteau's reagent solution in distilled water (1/13) (Pastoriza et al., 2011). The reagents were mixed, allowed to stand for 3 min and then neutralized with 100 μL of 7% Na_2CO_3. The mixture was vortexed for 90 min and the absorbance was read at 765 nm in the FLUOstar Omega reader. The TPC was calculated using GA calibration curve and expressed in mg GAE/g DWE or DWP.

3.4. Measurement of antioxidant capacity of solid substances by QUENCHER procedure

The measurements of the total antioxidant capacity using modified ABTS•+, ORAC and TPC methods were applied directly to the solid ground material (Singleton & Rossi et al., 1965). In principle, all assays were carried in the same way as described for the extracts using 10 mg of the powdered sample. In ABTS•+ scavenging assay the sample was diluted with 40 μL methanol, 1460 μL ABTS•+ reagent added, vortexed for 90 min, centrifuged at 10500×g for 10 min, and 300 μL of optically clear supernatant was transferred to the micro plate.

In ORAC assay the reaction was started by adding to the sample 1.5 mL of fluorescein. The mixture was shaken for 90 min and then 175 μL of prepared solution was transferred to the micro plate incubated for 15 min at 37 °C and 25 μL of AAPH solution added.

For TPC, the sample was transferred to test tube with 40 μL of ET, 950 μL Folin–Ciocalteau's reagent solution. The reagents were mixed and allowed to stand for 3 min. Then the mixture was neutralized with 500 μL of 7 % Na_2CO_3, vortexed for 90 min and centrifuged at 10500 g for 10 min; the absorbance was measured at 765 nm.

In all methods, when the samples exerted too high antioxidant activity, they were diluted with microcrystalline cellulose as an inert material. Cellulose–reagent mixtures were used as blanks in all measurements. The results are expressed in μmol TE/g DWP (ORAC and ABTS•+) and mg GAE/g DWP (TPC).

3.5. UPLC/ESI–QTOF–MS analysis

An Acquity UPLC system with a binary solvent delivery system, an auto sampler with a 10 μL sample loop, a photodiode array (PDA) detector, a column manager, and a data station running the Compass acquisition and data software (Waters, Milford, MA, USA) combined with a Bruker maXis UHR–TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) was used. An Acquity HSS T3 column (1.7 μm, 50 × 2.1 mm, i.d.) was used for separation of compounds at 25 °C.

The mobile phase **for golden rod leaves and buckwheat flowers extract analysis** was initially composed of 60% eluent A (1%, v/v, formic acid solution in ultra pure water) and 40% B (acetonitrile), followed by a linear gradient from 10 to 20% of eluent B in 1.2 min, and later on, to 30% B in the following 1.8 min. Finally,

in the following 2.5 min the percentage of B was increased to 100%, and it was kept at these conditions during the following 0.5 min.

For bergenia roots and leaves extract analysis it was as follows: the mobile phase was initially composed of 90% eluent A (0.1%, v/v, formic acid solution in ultrapure water) and 10% B (acetonitrile), followed by a linear gradient from 10 to 20% of eluent B in 1.2 min, and later on, to 30% B in the following 1.8 min. Finally, in the following 2.5 min the percentage of B was increased to 100%, and it was kept at these conditions during the following 0.5 min.

After the analysis, the initial conditions were reintroduced over 1 min. Before each new run column was equilibrated for 2 min. The flow rate was 0.4 mL/min and the effluents were monitored at 254 nm. The effluents from the PDA detector were introduced directly into the UHR–QTOF mass spectrometer equipped with an ESI source. Instrument control and data acquisition were achieved using the Compass 1.3 (HyStar 3.2 SR2) software. MS experiments were performed in negative ionization mode, the capillary voltage was maintained at +4500 V with the end plate offset at −500 V. Nitrogen was used as the drying and nebulising gases at a flow rate of 10.0 L/min and a pressure of 2.0 bar, respectively. Mass spectra were recorded in a range from 100 to 1200 m/z, at a rate of 2.5 Hz. Peak identification was carried out by comparing the retention times with those of the corresponding peaks in chromatograms of standards and/or by their accurate masses.

3.6. Determination of tocopherols by high performance liquid chromatography (HPLC)

Perkin Elmer Series 200 HPLC system was equipped with C_{30} reverse–phase column (particle size 5 μm, 250×4.6 mm) and thermostated at 30°C applying isocratic elution with acetonitrile/methanol/dichloromethane (72/22/6, v/v/v). Injection volume was 20 μL and flow rate 1 mL/min. Tocopherols were detected using fluorescence detector at 290 nm excitation and 330 nm emission; the analytes eluted in 20 min: α–T at 12.2 min, β–T at 10.6 min, δ–T at 8.9 min and γ–T at 10.2 min. Tocopherols were identified by comparing the retention time of peaks to those of pure standard solutions, which were prepared at different concentrations using mobile phase; for tocopherols 0–10 μg/mL. Extracts were prepared dissolving in mobile phase at final concentration 0.5 mg/mL. The calibration curves (peak area versus injected amount) were used to determine the quantity of tocopherols in the samples. Analyses were performed in triplicate.

3.7. Measurement of the effect of extracts on oil oxidation

3.7.1. Measurement of the effect of extracts on oil oxidation in Oxipres apparatus

The samples were prepared by mixing rapeseed oil (RO) with 0.5 % HX and CO_2 extracts and o/w (70/30) emulsions (EM) with 0.5% AC, ET/W and W extracts using sonication. Five grams of prepared sample were placed in a reactor of Oxipres apparatus (Mikrolab, Aarhus, Denmark) and thermostated at 120°C under oxygen atmosphere (0.5 MPa). Pressure changes, which occur due to the absorption of

oxygen consumed for oil oxidation, were recorded. The protection factor (PF) value of RO in case of using plants extracts and their antioxidant activity (AA) were calculated by the following formula: $PF = IP_X/IP_K$; where IP_X is induction period of a sample with additive (h), IP_K is induction period of a sample without additive (h). Each measurement was done in triplicate.

3.7.2. Oil and emulsions oxidation measurement in Rancimat apparatus

Plant extract's AA in RO and EM was also evaluated in a Metrohm 873 Biodiesel Rancimat apparatus (Metrohm Ltd., Herisau, Switzerland), which is measuring the increase of electrical conductivity due to the formation of volatile dicarboxylic acids in the course of lipid oxidation. The determination was performed at 120 °C, with an air flow rate of 20 L/h, using 5 g of RO/EM and 60 mL of distilled water in the flasks containing electrodes. The PFs were calculated as described above for Oxipres.

3.8. Statistical analysis

All analyses were carried out in triplicate and the results are expressed as a mean value ± standard deviation (SD). Significant differences among means were determined by one–way ANOVA, using the statistical package GraphPad Prism 5. Tukey's Least Significant difference (LSD) was used to determine significant difference among the treatments at $p < 0.05$. Correlation coefficients were calculated between each of the variables. Statistical difference was established at $p<0.05$. Correlation coefficients for antioxidant assays comparisons were calculated using MS Excel 2010.

IV. RESULTS AND DISCUSION

4.1. Biorefining of *Bergenia crassifolia* L. roots and leaves by high pressure extraction methods and evaluation of antioxidant properties and main phytochemicals in extracts and plant material

4.1.1. Yields of *Bergenia crassifolia* L. extracts

Botanicals are very complex materials containing various polarity soluble and insoluble constituents. Therefore, elaboration of fractionation schemes may provide the substances with different properties, which may be further adapted for the specific needs of the variety of products. Therefore, the yields of fractions are very important, particularly for the commercialization of the processes. For instance, agrorefinery of tansy by using traditional distillation/extraction methods produced 4 fractions with the yields 0.47, 2.15, 4.26 and 22.96%, possessing different antioxidant activities and consisting of various phytochemicals (Baranauskienė et al., 2014).

First step in *B. crassifolia* fractionation was intended isolating low polarity lipophilic molecules. Such molecules are of a particular interest for cosmetic industry. The yields of extracts obtained by nonpolar solvents in PLE and SFE–

CO_2 were in the following ranges: for BR from 0.15 (PLE_{70}–HX) to 0.61 (PLE_{140}–HX), for BL from 5.2 (CO_2) to 10.3 (PLE_{140}–HX). Adding 10% of co–solvent ethanol in SFE–CO_2 increased extract yields from BR and BL up to 0.71 and 8.15%, respectively (**Table 4.1**). It is interesting noting that raising PLE temperature from 70 to 140 °C enabled to considerably increase lipophilic fraction yield, although in case of BR it remained < 1%.

AC was particularly efficient solvent for BR, yielding more than 40% of extract from the defatted plant material, whereas in case of BL, the yields of this fraction were in the range of 4.71–11.4% DWR and 4.47–10.2% DWP, which is remarkably lower comparing to the yields obtained from the BL extraction residues with protic solvents, ET/W and W (10.2–22.98% DWR and 7.95–17.1% DWP). Previously performed studies, which applied traditional extraction methods, demonstrated that the yields of extracts as well as their antioxidant capacities highly depend on the plant species, extraction method and solvent polarity. For instance, acetone gave >3–fold higher yields than methanol from swallow–wort (Šliumpaitė et al., 2013a), while in case of woody betony acetone extract yield was almost 3–fold lower than methanol extract (Šliumpaitė et al., 2013b). It is interesting noting that in BR PLE raising temperature to 140 °C did not have significant effect ($p < 0.05$) on AC extract yield, however AC fraction yield in PLE was significantly higher when the residue of SFE–CO_2 was re–extracted. In case of BL the opposite results were obtained: increasing temperature doubled AC extract yield, while it remained almost similar both from the residues after PLE–HX and SFE–CO_2.

Table 4. 1. The yields % (w/w) and antioxidant characteristics of *B. crassifolia* roots and leaves extracted with different polarity solvents; TEAC (ABTS) and ORAC values are expressed in μmol TE/g, TPC in mg GAE/g in extract (E) and plant residues (DWR) and plant material (DWP) dry weight (DW).

Extraction method	Solvent	Yield	TPC			TEAC (ABTS)			ORAC		
		DWR	DWP	E	DWR	DWP	E	DWR	DWP	E	DWR	DWP
						Roots						
PLE_{70}	HX	0.15±0.12^{aA}	0.15	–	–		–	–		1315±119^{aA}	1.97^{aA}	1.97
	AC	41.2±3.0^{d}	41.1	187±4.80^{c}	76.8^{e}	76.9	2582±127^{d}	1064^{d}	1061	2332±208^{c}	961^{d}	958
	ET/W	19.2±1.1^{c}	11.3	167±4.91^{b}	32.0^{c}	18.9	1848±63.0^{c}	355^{b}	209	2583±182^{c}	496^{c}	292
	W	10.8±1.4^{b}	4.3	133±1.51^{a}	14.3^{a}	5.72	1388±98.1^{b}	150^{a}	150	1182±73.1^{a}	128^{a}	50.8
PLE_{140}	HX	0.61±0.0^{aC}	0.61	–	–		–	–		1267±16.4^{aA}	7.73.^{aC}	7.73
	AC	40.9±0.4^{d}	40.7	192±8.02^{c}	78.3^{e}	78.1	2422±63.1^{d}	991^{d}	986	2602±175^{c}	1064de	1059
	ET/W	39.6±1.5^{d}	23.2	153±6.44^{b}	60.6^{d}	35.5	1919±56.1^{c}	760^{c}	445	2258±203^{bc}	894^{d}	524
	W	10.2±1.9^{b}	1.93	125±5.81^{a}	12.7^{a}	2.41	1309±125^{b}	134^{a}	25.3	1281±111^{a}	131^{a}	24.7
$SFE–CO_2$	CO_2	0.37±0.1^{aB}	0.37	–	–		–	–		1356±37.1^{aA}	5.01^{aB}	5.01
	CO_2–ET	0.71±0.1^{aC}	0.71	–	–		–	–		2540±157^{cB}	18.0^{aD}	18.0
$SFE–CO_2$/ PLE_{70}	AC	46.6±1.3^{e}	46.4	195±10.50^{c}	90.9^{f}	90.5	2964±120^{e}	1381^{e}	1375	2539±250^{c}	1183^{e}	1178
	ET/W	15.6±1.5^{c}	8.27	161±3.71^{b}	25.1^{b}	13.3	1897±38.7^{c}	296^{b}	157	2100±75.1^{bc}	328^{b}	174
$SFE–CO_2$/ PLE_{140}	W	10.1±0.1^{b}	3.78	115±7.04^{a}	11.6^{a}	4.35	882±65.1^{a}	89.1^{a}	33.3	934±24.1^{a}	94.3^{a}	35.3

Table 4.1. (continued)

Leaves												
PLE_{70}	HX	$5.72±0.50^{aA}$	5.72	–	–		–	–		$233±2.14^{aA}$	13.3^{aA}	13.3
	AC	$5.50±0.11^{a}$	5.18	$183±14.7^{c}$	10.1^{a}	9.48	$1987±154^{bc}$	111^{a}	103	$2193±179^{e}$	121^{b}	114
	ET/W	$10.8±0.34^{c}$	9.59	$207±10.1^{c}$	22.4^{c}	19.9	$2214±123^{bc}$	239^{c}	212	$2574±212^{f}$	278^{cd}	247
	W	$10.2±1.04^{c}$	7.95	$152±10.2^{bc}$	15.5^{bc}	12.1	$1720±17.6^{b}$	175^{b}	137	$1610±123^{d}$	164^{b}	128
PLE_{140}	HX	$10.3±0.31^{cC}$	10.3	–	–		–	–		$304±26.3^{aA}$	31.3^{aC}	31.3
	AC	$11.4±1.00^{c}$	10.2	$197±13.8^{c}$	22.5	20.1	$1582±61.1^{b}$	180^{b}	161	$2100±99.0^{e}$	239^{c}	214
	ET/W	$21.9±1.27^{e}$	17.1	$218±16.5^{cd}$	47.7	37.3	$2000±189^{bc}$	438^{e}	342	$1957±45.5^{e}$	429^{e}	335
	W	$19.4±0.77^{e}$	10.9	$128±7.81^{ab}$	24.8	14.0	$1596±40.1^{b}$	309^{d}	174	$1065±46.3^{b}$	207^{c}	116
$SFE–CO_2$	CO_2	$5.2±0.21^{aA}$	5.2	–	–		–	–		$399±30.7^{aAB}$	20.7^{aB}	20.7
	CO_2–ET*	$8.15±0.14^{bB}$	8.15	–	–		–	–		$1386±47.1^{cdC}$	113^{cD}	113
$SFE–CO_2$/ PLE_{70}	AC	$4.71±0.27^{a}$	4.47	$180±13.4^{bc}$	8.48^{a}	8.05	$1851±100^{b}$	87.2^{a}	82.7	$2001±141^{e}$	94.2^{b}	89.4
	ET/W	$15.4±0.11^{d}$	13.9	$223±11.7^{cd}$	34.3^{d}	31.0	$2302±94.4^{bc}$	355^{d}	320	$2188±64.1^{e}$	337^{d}	304
$SFE–CO_2$/ PLE_{140}	W	$22.9±0.58^{e}$	17.1	$104±8.91^{a}$	23.8^{cd}	17.8	$1116±58.8^{a}$	256^{c}	191	$1159±88.6^{bc}$	265^{cd}	198

Values represented as mean ± standard deviation (n=3); different lowercase superscript letters within the same column (separately for roots and leaves) indicate significant differences at $p<0.05$; different uppercase superscript letters within the same column (separately for roots and leaves) indicate significant differences between lipophilic extracts at $p<0.05$. HX–hexane; AC–acetone; ET/W–ethanol/water (80/20); W–water; CO_2–ET–carbon dioxide with co–solvent ethanol. *$SFE–CO_2$ with co – solvent ethanol was performed separately from the whole plant material and is not included in to the total content plant material and is not included in to the total content.

Finally, the highest polarity solvents, ET/W and W, were applied. In PLE, raising temperature doubled ET/W extract yield, while in case of SFE–CO_2/PLE–AC residue it was lower than after PLE–HX/AC extraction. Similar tendencies were observed for BL in PLE; however, SFE–CO_2/PLE–AC residue gave higher ET/W extract yields comparing to PLE–HX/AC. Water additionally extracted slightly that 10% from BR residues (1.93–4.30% DWP) at all applied schemes, while in case of BL, W fraction yield was approx. 2–fold (from DWR) and 1.37 times (from DWP) higher at 140 °C than at 70 °C. However, the highest yield of W extract was obtained during PLE of SFE–CO_2 residues, 22.9 and 17.1% from DWR and DWP, respectively. In general, the application of different processing schemes demonstrated that the total yields (sums of all extractions) from *B. crassifolia* were very high, e.g. in case of PLE at 140 °C it was 66.44% DWP for roots and 48.5% DWP for leaves (**Fig. 4.2**).

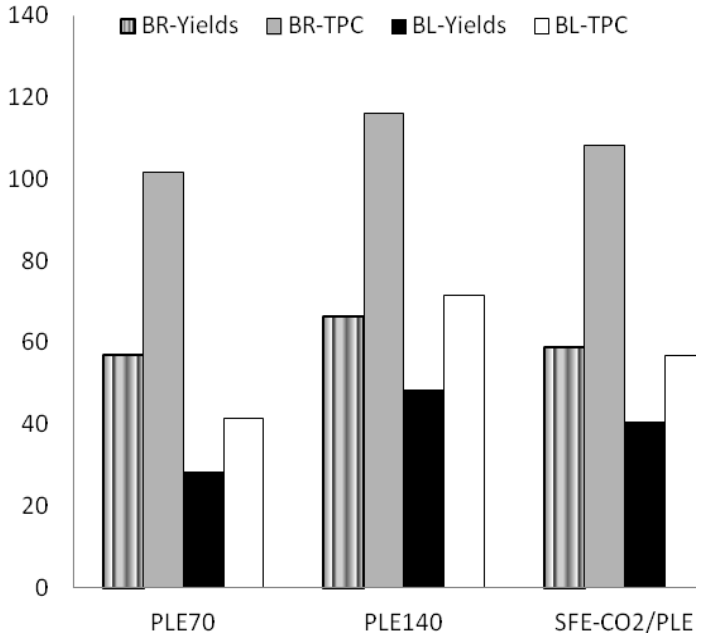


Fig. 4.2. Total yield (%) and of extracts isolated by all solvents and total phenolic content (TPC, GAE/g DW) in plant material of *B. crassifolia* L. roots (BR) and leaves (BL).

4.1.2. Total phenolic content and antioxidative properties of *Bergenia crassifolia* extracts

There are many assays for the *in vitro* assessment of antioxidant properties of plant extracts, the majority of them are based on electron/hydrogen atom transfer reactions. Huang et al. (2005) concluded that ORAC, TPC measured with Folin–Ciocalteu reagent and one of the electron/hydrogen transfer assays should be recommended for the representative evaluation of antioxidant properties. Electron transfer based methods include the TPC assay with Folin–Ciocalteu reagent and

TEAC measurement by the $ABTS^{\bullet+}$ decolourisation assay. All these methods were applied for assessing antioxidant potential of *B. crassifolia* L. in our study. PLE with different polarity solvents (HX, AC, ET/W (80/20) and W) and SFE–CO_2 with and without a co–solvent ET were applied for the extraction of active compounds from *B. crassifolia* L. leaves (BL) and roots (BR).

There were remarkable variations in the antioxidant capacity values between different anatomical parts of *B. Crassifolia* L., applied solvent and assay procedure; for instance TPC in BR and BL extracts was in the ranges of 115–195 and 104–223 mg GAE/g, respectively (Table 1). It may be observed that TEAC and TPC values of BR extracts gradually decreased during extraction with AC, ET/W, W and SFE–CO_2. Lipophilic fractions isolated with HX and CO_2 were not used in these assays due to their poor solubility in the reaction media. All analysed BR extracts were strong antioxidants in ORAC assay (934–2602 μmol TE/g).

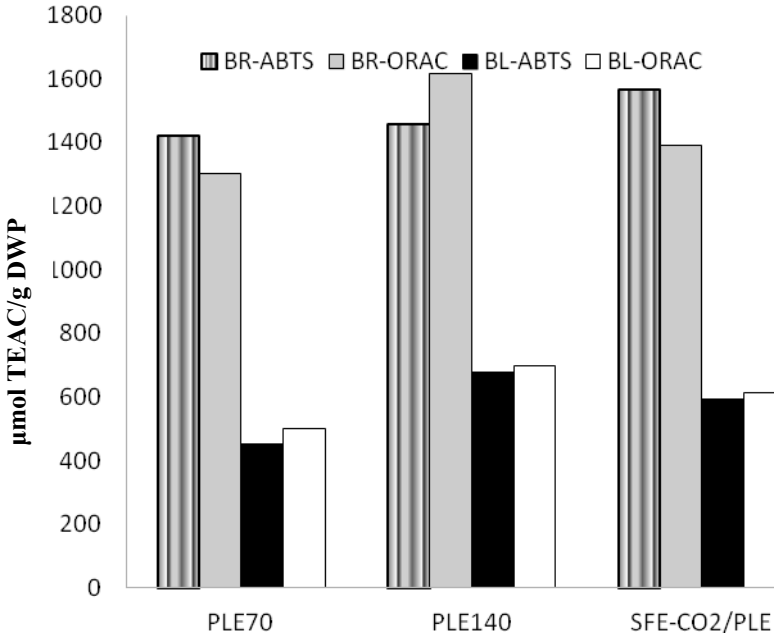


Fig. 4.3. Total TEAC (ABTS) and ORAC values, in μmol TEAC/g DWP of *B. crassifolia* roots (BR) and leaves (BL), obtained using all extraction solvents.

It is interesting noting that in case of PLE at 70 and 140 °C ORAC of ET/W extracts was approx. 2–fold stronger than W extract. The values were also calculated for dry plant materials (DWR and DWP); they are important as showing how much of antioxidatively active substances may be isolated from the plant material by each fractionation step. It may be observed that AC isolated the major part of antioxidants from BR, followed by ET/W and W. For instance, in PLE at 70 °C AC, ET/W and W isolated 75.7, 18.6 and 5.63% of the total TPC measured in BR DWP. This tendency agrees with phytochemical composition data for BR (**Table 4.1**). The highest amounts of TPC isolated during consecutive extractions from BR and BL

were 116 and 71.4 mg/g DWP (**Fig. 4.2**). The highest total values of ORAC in BR, ORAC and TEAC in BL DWP were obtained by the consecutive PLE at 140 °C; while the highest TEAC value in BR DWP was obtained in case of SFE–CO_2–PLE (**Fig. 4.3**).

It should be mentioned, that temperature had significant effects for all BL extracts in obtained yields and antioxidant capacity values in TPC (ET/W; W), $ABTS^{\bullet+}$(AC) and ORAC (ET/W; W) assays ($p > 0.05$). In fact, the results obtained clearly demonstrate that several extraction steps using different polarity solvents would be required for exhaustive isolation of antioxidants both from the *B. crassifolia* roots and leaves.

A strong correlation between TPC and RSC was observed: for instance, in case of BR TPC *vs* $ABTS^{\bullet+}$, R^2= 0.97; TPC *vs.* ORAC, R^2= 0.92. These findings reveal the compositional complexity of the antioxidatively active constituents in different anatomical parts of *B. crassifolia*. Since TPC showed high correlation with TEAC and ORAC it may be concluded that the substances reacting with Folin–Ciocalteu's reagent are a good predictors of the *in vitro* antioxidant activity for *B. crassifolia* extracts.

Antioxidant properties of BL were studied previously by using different methods, however the results reported in published articles are difficult to compare. For instance, Hendrychová et al. (2014) reported that water extracts of *B. crassifolia* and *B. x ornata* were the most active radical scavengers in $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays. Antioxidant activity correlated well with the content of total tannin, especially in the $ABTS^{\bullet+}$ assay, which suggests an important role of these compounds as antioxidants. It was also shown that phenolics in BL were dependent on seasonal factors; a significant correlation was found between arbutin and tannin contents and the average humidity. In other studies Shikov et al. (2012) reported TPC in water extracts of black and fermented *B. crasiffolia* leaves 32.51 and 57.2 mg GAE/g DW, respectively, while Ivanov et al. (2011) measured RSC of different *B. crassifolia* rhizome extracts in $DPPH^{\bullet}$ assay; depending on extraction solvent the IC_{50} values were 2.9–6.11 μg/mL.

4.1.3. Direct evaluation of antioxidant capacity of solid substances by QUENCHER method

It is known that some insoluble antioxidatively active constituents may be strongly bound to other components in plant material matrix and therefore are not extracted by solvents. QUENCHER procedure was elaborated in order to measure antioxidant activity of the whole plant material including its insoluble fraction (Serpen et al., 2007). For this part of the study only environmentally friendly and cheap solvents, CO_2 and W were applied. It may be observed that in PLE all antioxidant potential indicators gradually decreased; slightly lower values were determined for the residues after PLE at 140 °C, except for TEAC. For instance, TPC in the PLE residues constituted 35–43 and 31–33% of the initial values of BR and BL, respectively. In case of SFE–CO_2 the TPC in the extraction residue was found even higher than in the initial BR material. Polyphenolic compounds are

poorly soluble in CO_2 and, most likely, treatment of BR at high pressure during SFE–CO_2 resulted in some changes, which made some antioxidatively active groups better accessible in the reaction with Folin–Ciocalteu reagent. The co–solvent ethanol significantly reduced TPC value in BL ($p < 0.05$), while in case of BR the effect was not significant.

Table 4.2. Antioxidant characteristics of solid substances of *B. crassifolia* leaves and roots measured by QUENCHER method; TPC expressed as mg GAE, TEAC and ORAC μmol TE in 1 g DWP.

Sample	Solvent	TPC	TEAC (ABTS)	ORAC
		Roots		
Before extraction	–	224 ± 21.2^{b}	1875 ± 52.1^{c}	2974 ± 185^{c}
After PLE_{70}	W	96.7 ± 8.21^{a}	260 ± 14.4^{a}	1601 ± 165^{b}
After PLE_{140}	W	78.2 ± 8.07^{a}	264 ± 41.7^{a}	1006 ± 143^{a}
After SFE–CO_2	CO_2	289 ± 25.5^{c}	1836 ± 132^{c}	2855 ± 204^{c}
	CO_2–ET	211 ± 24.1^{b}	1480 ± 154^{b}	2883 ± 159^{c}
After SFE–CO_2/ PLE_{140}	W	89.0 ± 10.9^{a}	235 ± 21.4^{a}	1600 ± 168^{b}
		Leaves		
Before extraction	–	182 ± 12.7^{c}	668 ± 71.2^{b}	
After PLE_{70}	W	59.7 ± 7.71^{a}	114 ± 10.7^{a}	
After PLE_{140}	W	56.1 ± 8.01^{a}	136 ± 4.07^{a}	
After SFE–CO_2	CO_2	167 ± 17.2^{c}	854 ± 95.7^{b}	
	CO_2–ET	108 ± 19.5^{b}	1001 ± 124^{bc}	
After SFE–CO_2/ PLE_{140}	W	53.9 ± 6.11^{a}	135 ± 14.1^{a}	

Values represented as mean ± standard deviation (n=3); different superscript letters within the same column (separately for roots and leaves) indicate significant differences at $p<0.05$. HX–hexane; AC–acetone; ET/W–ethanol/water (80:20); W–water; CO2–ET–carbon dioxide with co–solvent ethanol.

It may be observed that $ABTS^{\bullet+}$ QUENCHER values for the leaves were higher than for the roots except for some PLE residues extracted with ET/W and W, when TPC and ORAC values were very different (**Table 4.2**). Comparing QUENCHER results with those obtained by analysing the extracts some interesting observations can be observed. The sum of RSC values of roots and leaves obtained in TPC, $ABTS^{\bullet+}$ and ORAC assays by analysing the extracts isolated by serial extraction and calculated 1 g DW DWP was comparable to the values obtained for the initial material by QUENCHER assay. For instance, TPC QUENCHER value of BR before extraction was 224 mg GAE/g DW, while the sum of TPC isolated by the increasing polarity solvents (Table 1, DWP) and remaining in the residue after PLE at 140 °C (Table 2) was only by 13% lower, 194.2 mg GAE/g DW

(Σ_{TPC} = 78.1 + 35.5 + 2.41 + 78.2). Several reasons may be considered for explaining these findings. First of all, using the series of sequential extractions the majority of active compounds were isolated in previous steps of extraction. Another reason, as it was already mentioned, some part of antioxidatively active compounds may remain in the matrix after extraction because they are bound to other constituents. Consequently, application of antioxidant assays to the extracts by traditional methods and to the solid substances by QUENCHER procedure enables to characterise antioxidative potential of plant material and the effectiveness of various separation processes more comprehensively. To the best of our knowledge this approach has never been applied for evaluating *B. crassifolia*; moreover, it was not reported previously for other botanicals as well.

4.1.4. Characterization of phytochemicals by chromatography–mass spectrometry

Phytochemical composition of extracts was analysed by UPLC–QTOF–MS and the following main compounds were identified in BR and BL extracts by measuring their accurate mass and retention time: bergenin, catechin gallate, ellagic acid and quercetin–3–β–D–glucoside (**Fig. 4.4**). On the other hand, a large number of recorded peaks on the chromatograms indicate that the extracts are complex mixtures of compounds; however, exact mass data obtained by UPLC–QTOF–MS was not sufficient for their identification, because mass spectra libraries give too many candidate structures for the measured masses. On the other hand, identification of minor components was beyond the scope of the present study; the main focus was on major and most important constituents in terms of possible commercial applications.

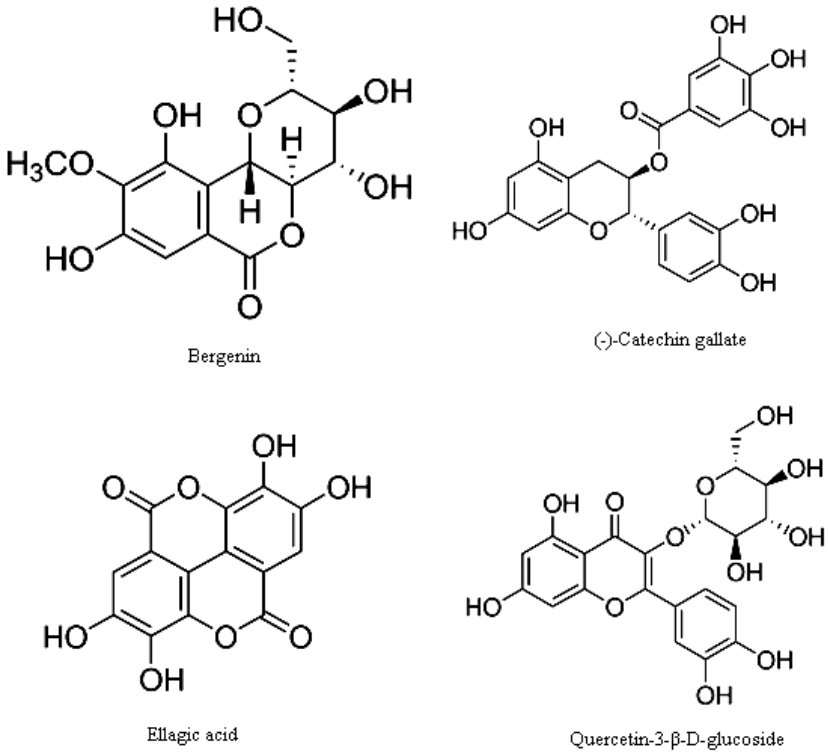


Fig. 4.4. Structures of the main compounds quantified in *B. crassifolia* L. extracts.

It may be clearly observed that bergenin was the major quantitatively constituent (**Table 4.3.**) both in BR and BL, however the total amount of extracted bergenin from the roots (45.24–50.35 mg/g DWP) was remarkably higher than from the leaves (3.69–4.51 mg/g DWP). Bergenin is one of active ingredients in herbal and Ayurvedic formulations exhibiting antiviral, antifungal, antitussive, antiplasmodial, antiinflammatory, antihepatotoxic, antiarrhythmic, antitumor, antiulcerogenic, antidiabetic and wound healing properties (Bajracharya, 2015). The presence of a large percentage of bergenin was linked to the radical scavenging activity measured by the FRAP and NADH assays (Hendrychová et al., 2015). The content of quercetin–3–β–D–glucoside and catechin gallate was also considerably higher in BR than in BL, while ellagic acid was detected only in BL. It may be observed that bergenin was found in reasonable amounts in all fractions except for HX and SFE–CO_2 extracts of BL.

Table 4.3 Concentration of major phenolic compounds in *Bergenia crassifolia* extracts (E) and plant residues after extraction (DWR) and in plant material (DWP), mg/g

Sample	Solvent	Bergenin			(–)–Catechin gallate			Ellagic acid			Quercetin–3–β–D–glucoside		
		E	PR	DWP	E	DWR	DWP	E	DWR	DWP	E	DWR	DWP
							Roots						
PLE 70°C	HX	75.7±2.0	0.11	0.11	0.12±0.00	<0.01		–	–		–	–	
	AC	81.3±4.2	33.5	33.4	16.2±0.10	6.67	6.66	–	–		21.3±0.1	8.78	8.75
	ET/W	74.9±3.8	14.4	8.46	12.4±0.24	2.38	1.40	–	–		16.6±0.3	3.19	1.88
	W	76.1±2.4	8.22	3.27	–	–		–	–		–	–	
Total				**45.24**			**8.06**						**10.63**
PLE 140°C	HX	63.0±2.4	0.38	0.38	–	–		–	–		–	–	
	AC	76.6±3.1	31.3	31.2	16.8±0.91	6.87	6.84	–	–		27.4±1.0	11.2	11.2
	ET/W	75.5±4.5	29.9	17.5	11.0±0.12	4.35	2.55	–	–		15.7±0.6	6.22	3.64
	W	65.8±3.1	6.71	1.27	–	–		–	–		–	–	
Total				**50.35**			**9.39**						**14.84**
SFE–CO_2	CO_2–ET*	130±7.1	0.92	0.92	0.03±0.00	<0.01	<0.01	–	–		–	–	
	CO_2	56.5±2.5	0.21	0.21	–	–		–	–		–	–	
SFE–CO_2/ PLE70°C	AC	92.6±5.6	43.2	43.0	16.2±0.01	7.55	7.52	–	–		21.5±0.9	10.02	10.02
	ET/W	46.0±1.8	7.18	3.80	5.81±0.05	0.91	0.48	–	–		1.84±0.0	0.29	0.15
SFE–CO_2/ PLE140°C	W	45.4±2.2	4.59	1.72	–	–		–	–		–	–	
Total				**49.65**			**8.00**						**10.17**

Table 4.3. (continued)

					Leaves								
	HX	–	–			–			–		–	–	
PLE 70 °C	AC	21.2±1.1	1.17	1.10	0.17±0.00	0.01	0.01	0.04±0.00	<0.01	<0.01	35.1±1.0	1.93	1.82
	ET/W	16.5±0.1	1.78	1.58	0.12±0.00	0.01	0.01	0.04±0.00	<0.01	<0.01	27.5±1.0	2.97	2.64
	W	12.7±0.4	1.30	1.01		–		2.56±0.01	0.26	0.20	–	–	
Total				**3.69**			**0.02**			**0.20**			**4.46**
	HX		–			–			–		–	–	
PLE 140°C	AC	16.7±0.1	1.90	1.70	0.16±0.00	0.02	0.02	0.06±0.00	0.01	0.01	24.2±1.0	2.76	2.47
	ET/W	10.2±0.5	2.23	1.04	0.25±0.01	0.06	0.04	0.07±0.00	0.02	0.01	8.57±0.0	1.88	1.47
	W	7.35±0.0	1.43	1.26	–	–		–	–			–	
Total				**4.00**			**0.06**			**0.02**			**3.94**
SFE–CO_2	CO_2–ET*	89.0±4.2	7.25	7.25	–	–		–	–		–	–	
	CO_2	–	–		–	–		–	–		–	–	
SFE–CO_2/ PLE70°C	AC	28.5±1.1	1.34	1.27	0.33±0.01	0.016	0.015	0.05±0.00	<0.01		43.5±1.2	2.05	1.94
	ET/W	12.3±0.1	1.89	1.71	0.05±0.00	0.008	0.007	0.07±0.00	0.01	0.01	8.89±0.0	1.37	1.24
SFE–CO_2/ PLE140°C	W	8.96±0.1	2.05	1.53	–	–		–	–		–	–	
Total				**4.51**			**0.022**			**0.01**			**3.18**

Values represented as mean ± standard deviation (n = 3); DW–dry weight; HX–hexane; AC–acetone; ET/W–ethanol/water (80:20); W–water; CO2–ET–carbon dioxide with 10% of ethanol. *SFE–CO2 with co–solvent ethanol was performed from the whole plant material and is not included in to the total content.

Particularly high concentrations of bergenin were determined in SFE–CO_2–ET extracts, however, the yield of this fraction from BR was very low (0.71%) and therefore this extraction procedure enabled to isolate only a small part of the total bergenin (0.92 mg/g DWP) present in BR. However, in case of leaves the yield of SFE–CO_2–ET extract was remarkably higher (8.15%) and, considering high concentration of bergenin (89 mg/g extract), this extraction method might be promising in the production of bergenin preparations from BL. AC due to a very high extract yields was the most efficient solvent in the extraction of bergenin and other major polyphenolics from BR; for instance, extraction step with AC gave 62.0–86.6%, 75.5–98.5% and 72.8–94.0% of the total bergenin, quercetin–3–β–D–glucoside and catechin gallate, respectively. However, in case of BL, ET/W was the most efficient solvent for bergenin, although it was extracted more evenly during all process steps with all solvents, except for HX and CO_2. It may be also observed that the efficiency of isolation of the quantified constituents from BR was higher in PLE_{140} the total content of extracted bergenin, quercetin–3–β–D–glucoside and catechin gallate was higher by 11.3, 39.6 and 16.5%, respectively, comparing to PLE_{70}. In general, these findings show that the major bioactive constituents of BR might be more strongly embedded in the tough solid plant particles and they remain stable at high temperature, which is important in selecting a proper extraction/fractionation procedure. The effect of temperature on the extraction of bergenin and quercetin–3–β–D–glucoside from BL was not so important, while the total content of ellagic acid extracted at 140 °C was many times lower than at 70 °C. Most likely, ellagic acid becomes unstable at high temperature. Consequently, extraction of BL should be preferably performed at lower temperatures.

Various phenolic compounds and flavonoids were reported in *B. crassifolia* previously: for instance, arbutin and bergenin and ellagic gallic acid were reported in BL as the main constituents, while protocatechuic acids and hydroquinone as other important compounds (Shikov et al., 2010, Shikov et al., 2012 and Pozharitskaya et al., 2007). Golovchenko et al. (2007), isolated a pectin, polysaccharide, named bergenan from the freshly collected BL by extraction with an aqueous solution of ammonium oxalate.

4.1.5. Effect of *B. crassifolia* L. extracts on rapeseed oil and emulsions oxidation

Lipid oxidation is a complex phenomenon induced by oxygen in the presence of initiators, particularly at higher temperatures. The change of oxygen pressure in the reaction vessel at the end of the induction period, which indicates rapid formation of hydroperoxides, can be quite precisely measured by Oxipres method. The oil samples were prepared by mixing pure refined rape seed oil (RO) with 0.5% of selected additive by using sonication. The model emulsion system (EM) was produced using oil/water and (70/30) and 1% of Tween 40. The samples were prepared as described for RO. The oxidative stability of RO and EM with extracts was measured by instrumental Oxipres and Rancimat methods and the obtained results are expressed by the autoxidation induction period (IP) and protection factor

(PF) (**Table 4.4**). In general, all extracts demonstrated oil and emulsion stabilizing for oxidation effects: PF was in the range of 1.02–1.62. The highest antioxidative effects demonstrated AC and ET/W extracts of BR and BL; these extracts also demonstrated the highest effects against lipid oxidation: PF 1.38–1.58 for AC and 1.43–1.62 for ET/W.

Table 4.4. Antioxidant characteristics of *B. crassifolia* L. extracts (SFE and PLE in series extraction) in rapeseed oil (RO) and emulsion (EM) at 120 ˚C.

Extract additives	Oxipres				Rancimat	
	RO		EM		EM	
	IP	PF	IP	PF	IP	PF
Roots						
Control	2.18±0.01	1.00	2.64	1.00	9.24	1.00
$PLE_{70}HX$	2.63±0.01	1.21	–	–	–	–
CO_2	2.23±0.02	1.02	–	–	–	–
$PLE_{70}AC$	–	–	3.76±0.02	1.42	14.57±0.04	1.58
$PLE_{70}ET/W$	–	–	3.75±0.01	1.42	15.01±0.03	1.62
$PLE_{140}W$	–	–	3.25±0.01	1.23		
Leaves						
$PLE_{70}HX$	2.61±0.01	1.19	–	–	–	–
CO_2	2.26±0.01	1.04	–	–	–	–
$PLE_{70}AC$	–	–	3.14±0.02	1.18	12.76±0.02	1.38
$PLE_{70}ET/W$	–	–	3.29±0.03	1.25	13.21±0.01	1.43
$PLE_{140}W$	–	–	2.87±0.02	1.09	–	–

HX–hexane; CO2–carbon dioxide; AC–acetone; ET/W–ethanol/water (80/20); W–water; Values represented as mean ± standard deviation (n=3); IP–induction period; PF–protector factor.

4.1.6. Conclusions

Bergenia crassifolia L. roots and leaves were proved to be a good source of polyphenolic compounds with high antioxidant potential, which was demonstrated by the *in vitro* chemical assays and in vegetable oil. It was shown that application of supercritical fluid and pressurised liquid extraction schemes with different solvents and process parameters may provide several fractions, in total constituting > 66% of soluble substances from the roots and > 48 from the leaves. Four phenolic constituents were quantified in *B. crassifolia* extracts, bergenin being the major quantitatively constituent, followed by catechin gallate, ellagic acid and quercetin–3–β–D–glucoside. *B. crassifolia* roots were several times richer in the quantified phytochemicals than leaves. In general, the results obtained demonstrate that selection of proper biorefining schemes and parameters considerably assists in

valorising *B. crassifolia* as a promising industrial crop for developing various natural products.

4.2. Biorefining of buckwheat (*Fagopyrum esculentum* Moench.) flowers by supercritical fluid and pressurized liquid extraction and evaluation of antioxidant properties and main phytochemicals in fractions and plant material

4.2.1. The yields of fractions isolated from buckwheat flowers by different solvents and methods.

Botanicals are very complex biological structures composed of various groups of compounds. Therefore, proper selection and elaboration of extraction/fractionation schemes is an important task for obtaining the products of desirable properties and composition. Consecutive extraction with the increasing polarity solvents may be a good choice for the isolation of lipophilic and hydrophilic fractions from BWF and, to the best of our knowledge, such approach has not been applied previously to buckwheat. Moreover, the reports on consecutive application of SFE and PLE are very scarce to other botanical species as well.

It may be observed that the yields of lipophilic fractions isolated with HX or CO_2 were rather small (**Table 4.5**); however, PLE, independently on process temperature, gave almost 2–fold higher yields than SFE–CO_2. Application of 10 % co–solvent ethanol increased the yield in SFE–CO_2 by 48 %. The increasing polarity solvents, AC, ET/W and W gave remarkably higher yields, which were highly dependent on the temperature in PLE. Thus, the yields of AC, ET/W and W extracts calculated on the basis of DWP at 140 °C were by 60, 50.3 and 220.3 %, respectively higher than at 70 °C. The total extract yields in PLE_{70}, and PLE_{140} were 37.02 and 64.05 %, respectively. For comparison, Hinneburg & Neubert (2005) reported that hydroethanolic extract yields from buckwheat herb, depending mainly on extraction temperature, varied between 1.04 and 1.48 %, i.e. many times lower temperature was by 23 % higher as well as the W yield in PLE_{140} from SFE–CO_2 and W yield obtained during PLE at the same temperature.

4.2.2. Antioxidative properties of buckwheat flower extracts

Determination of antioxidant capacity of botanical extracts by the in vitro assays may be considered as a first step in the evaluation of their bioactivities. And although such indicators are not sufficiently informative in terms of their links with possible health effects they are widely used for the preliminary assessment.

Table 4.5. The yields % (w/w) and antioxidant capacity characteristics of *F. esculentum* flower extracts isolated by different solvents; TEAC (ABTS) and ORAC values are expressed in μmol TE/g, TPC in mg GAE/g in extract (E) and plant residues (DWR) and plant material (DWP) dry weight (DW).

Sample	Solvent	Yields		TPC			TEAC (ABTS)			ORAC		
		DWR	DWP	DWE	DWR	DWP	DWE	DWR	DWP	DWE	DWR	DWP
PLE_{70}	HX	3.10 ± 0.12^{aC}	3.10^{ab}							714 ± 25^{a}	22.1^{ab}	22.1^{ab}
	AC	12.2 ± 0.91^{c}	11.8^{b}	309 ± 17.2^{c}	37.7^{b}	36.5^{c}	2401 ± 100^{d}	293^{c}	283^{c}	2087 ± 110^{e}	255^{e}	246^{d}
	ET/W	21.7 ± 1.20^{d}	18.5^{c}	292 ± 19.3^{c}	63.4^{d}	54.0^{d}	2211 ± 142^{d}	480^{e}	409^{de}	2114 ± 100^{c}	459^{g}	391^{de}
	W	6.82 ± 0.74^{b}	4.54^{ab}	245 ± 17.0^{b}	16.7^{a}	11.1^{a}	1779 ± 95^{b}	121^{a}	80.8^{a}	1974 ± 125^{c}	135^{c}	89.6^{c}
	Σ		**37.02**			**101.6**			**772.8**			**748.7**
PLE_{140}	HX	3.15 ± 0.22^{aC}	3.15^{ab}	–	–	–	–	–	–	979 ± 47^{ab}	30.8^{b}	30.8^{b}
	AC	19.5 ± 1.01^{d}	18.9^{c}	228 ± 10.1^{b}	44.5^{bc}	43.1^{cd}	1797 ± 122^{b}	350^{d}	340^{d}	1514 ± 99^{b}	295^{ef}	286^{d}
	ET/W	35.6 ± 2.71^{f}	27.8^{e}	272 ± 15.0^{bc}	96.8^{e}	75.6^{f}	2124 ± 154^{d}	756^{g}	590^{e}	1002 ± 29^{ab}	357^{f}	279^{d}
	W	28.3 ± 1.88^{e}	14.2^{b}	189 ± 11.7^{ab}	53.5^{c}	26.8^{b}	1383 ± 84^{a}	391^{d}	196^{b}	651 ± 30^{a}	184^{d}	92.4^{c}
	Σ		**64.05**			**145.5**			**1126**			**688.2**
SFE	CO_2	1.78 ± 0.17^{aA}	1.78^{a}	–	–	–	–	–	–	807 ± 37^{ab}	14.4^{a}	14.4^{a}
SFE	CO_2/ET*	2.63 ± 0.11^{aB}	2.63^{ab}	–	–	–	–	–	–	1012 ± 56^{ab}	26.6^{ab}	26.6^{ab}
SFE–CO_2/PLE_{70}	AC	12.6 ± 1.04^{c}	12.4^{b}	312 ± 12.4^{c}	39.3^{b}	38.7^{c}	2197 ± 59^{d}	214^{b}	272^{c}	2071 ± 100^{c}	261^{e}	257^{d}
	ET/W	26.5 ± 1.54^{de}	22.7^{d}	265 ± 11.8^{bc}	70.2^{d}	60.2^{de}	2371 ± 102^{d}	628^{f}	538^{e}	2000 ± 158^{c}	530^{h}	454^{e}
SFE–CO_2/PLE_{140}	W	22.9 ± 0.50^{d}	14.5^{b}	174 ± 14.6^{a}	39.8^{b}	25.2^{b}	1237 ± 105^{a}	283^{c}	179^{b}	672 ± 32^{a}	154^{c}	97.4^{c}
	Σ		**51.38**			**124.1**			**989**			**822.8**

Values represented as mean ± standard deviation (n=3); different lowercase superscript letters within the same column indicate significant differences at $p<0.05$.

Different uppercase superscript letters within the same column indicate significant differences between lipophilic extracts at $p<0.05$.

HX–hexane; AC–acetone; ET/W–ethanol/water (80/20); W–water; CO2–ET–carbon dioxide with co–solvent ethanol.

*SFE–CO_2 with co–solvent ethanol was performed separately from the whole plant material and is not included in to the total content plant material and is not included in to the total content.

DWE – dry weight extract; DWR – dry weight extraction residue; DWP – dry weight initial plant material.

Isolation efficiency of phenolic compounds from plant material depends mainly on the sample matrix, their molecular structure (*e.g.* the number of aromatic rings and hydroxyl groups), polarity and concentration. Antioxidant activity indicators in our study were measured in different fractions and recalculated for the residues and plant material dry matter, DWR and DWP, respectively (**Table 4.5**). Both values are practically important as showing antioxidant potential of extracts and the effectiveness of a solvent, e.g. how much of antioxidatively active compounds selected solvent may isolate from the plant material. There were remarkable variations in the obtained values between applied solvents and assay procedures. For instance, antioxidant capacity values of the extracts isolated with the same solvent by PLE_{140} were lower than at PLE_{70} from 1.1 (TPC–PLE–ET/W) to 3.0 times (ORAC–PLE–W); however, due to remarkably higher yields the sum of TPC isolated from plant material was higher in case of PLE_{140}, 145.5 *vs* 101.6 GAE/g DWP. In case of PLE of SFE–CO_2 residues, the sum of TPC was 124.1 GAE/g DWP. Lower TPC values in high yield extracts may be explained by the dilution of antioxidatively active constituents with the neutral ones. Similar tendencies may be observed for ORAC and TEAC values; for instance, the extracts isolated in PLE_{140} were weaker antioxidants than in PLE_{70} measured in ABTS assay, however, the total TEAC values obtained at 140 °C was 1.46 times higher than at 70 °C. Only in case of lipophilic fraction, ORAC was approx. 1.4 times higher for the extract isolated by PLE_{140} with HX. It is also interesting noting the total SFE–CO_2–PLE ORAC value obtained for DWP was higher than in case of PLE performed at 70 and 140 °C. Application of the co–solvent ET in SFE–CO_2 enabled obtaining the extracts with higher ORAC values by 25 %. Comparing the solvents W extracts were weaker antioxidants, while AC and ET/W extracts were stronger antioxidants with some exceptions depending on the extraction temperature and assay method.

A strong correlation between TPC and other antioxidant activity assays was observed: TPC vs. TEAC, R^2=0.77 and TPC vs. ORAC, R^2=0.81; therefore, TPC measured by Folin–Ciocalteau's reagent may be accepted as a good predictor of the *in vitro* antioxidant activity for BWF extracts.

Antioxidant properties of buckwheat leaves, stems and flowers were studied previously by using different methods, however the results obtained are difficult to compare because in some studies they are expressed in the percentage of scavenged radicals by the selected extract concentration (Acar et al., 2011; Sytar, 2014). For instance, Sytar (2014a) showed that methanolic extracts of different types of buckwheat inflorescences are good radical scavengers; TPC values measured in this study were 56.3÷73.0 mg GAE/g DWP, which is remarkably lower comparing with the sum of TPC obtained during consecutive high pressure extractions with several solvents in our study. Holasova et al. (2002) also reported remarkable antioxidant activity of buckwheat leaves, although TPC was not high, 39.5 mg/g DWP. The content of TPC in the flowers of buckwheat cultivated in Turkey was even lower, 24.3 mg GAE/g DWP (Acar et al., 2011). Zielinska et al. (2012) reported that free radical scavenging potential of common and tartary buckwheat water extracts in $DPPH^{\bullet}$ assays, depending on flowering state, was from 492 to 675 μmol TE/g DWP,

which is lower compared to the sum of TEAC (772.8–1126 μmol TE/g DWP) and ORAC (688.2–822.8 μmol TE/g DWP) values determined in our study. These scarce reports on BWF antioxidant activity clearly show that systematic study was necessary for a more comprehensive evaluation of this plant material.

4.2.3. Direct evaluation of antioxidant capacity of solid plant material using QUENCHER procedure

It is known that some insoluble antioxidatively active constituents may be strongly bound to other components in plant material matrix and are not extracted by organic solvents or enzymatic treatments without altering their molecular nature.

Table 4.6. Antioxidant characteristics of solid substances of *F. esculentum* flowers measured by QUENCHER method; TPC expressed as mgGAE, TEAC and ORAC μmol TE in 1 g DWP.

Plant material	Solvent	TPC	ABTS	ORAC
Before extraction	–	250 ± 14.8^{ef}	1381 ± 100^{f}	1657 ± 124^{e}
After PLE_{70}	HX	255 ± 11.7^{ef}	1309 ± 97^{f}	1637 ± 142^{e}
	AC	197 ± 17.0^{d}	1027 ± 90^{e}	1335 ± 108^{de}
	ET/W	102 ± 10.5^{c}	814 ± 72^{de}	1057 ± 84^{cd}
	W	78.0 ± 7.0^{bc}	625 ± 56^{cd}	903 ± 75^{c}
After PLE_{140}	HX	286 ± 20.1^{f}	1093 ± 92^{ef}	1677 ± 127^{ef}
	AC	198 ± 17.5^{d}	1007 ± 99^{de}	1621 ± 143^{e}
	ET/W	117 ± 9.9^{c}	511 ± 43^{bc}	665 ± 59^{bc}
	W	27 ± 2.0^{a}	213 ± 19.1^{a}	334 ± 29^{a}
After SFE	CO_2	240 ± 17.5^{e}	1303 ± 90^{f}	1647 ± 122^{ef}
After SFE–CO_2/PLE_{140}	W	95 ± 4.2^{c}	506 ± 48^{bc}	685 ± 42^{bc}
After SFE–CO_2/ET	CO_2/ET	228 ± 17.7^{de}	1334 ± 107^{f}	1605 ± 131^{e}

Values represented as mean ± standard deviation (n=3); different superscript letters within the same indicate significant differences. (GraphPad Prism 5. Tukey's Least Significant difference (LSD), $p<0.05$).

HX–hexane; AC–acetone; ET/W–ethanol/water (80:20); W–water; CO_2–ET–carbon dioxide with co – solvent ethanol.

For instance, Guo et al. (2011) reported that bound phenolics and flavonoids constituted up to 6.5 and 24 %, respectively, from the total amounts of these groups of compounds in tartary buckwheat grains. QUENCHER procedure enables to measure antioxidant potential of the whole plant material including its insoluble fraction (Serpen et al., 2007). In our case, it was important to follow the changes of antioxidant indicators of plant material in the course of consecutive extractions (**Table 4.6).** It is interesting noting that TPC value of plant material before extraction was higher than the sum of TPC isolated by the consecutive extraction process (Table 1). Application of a non–polar solvent did not have any significant effect ($p>0.05$) on the antioxidant activity values of plant material.

The following reasons may be suggested: (i) (poly) phenolic antioxidants are not soluble in HX or CO_2 whereas lipophilic antioxidants constitute only a small fraction; (ii) bound antioxidatively active constituents become better available for HAT/SET reactions after removal of lipophilic substances from the plant matrix.

Consecutive extractions with the increasing polarity solvents gradually decreased antioxidant potential of extraction residues; however, the final residues were still antioxidatively active materials. Thus TPC, TEAC and ORAC values after the final step of PLE_{70} were lower comparing with the initial plant material 3.2, 2.2 and 1.8–fold, respectively. It may be observed that PLE_{140} most exhaustively isolated antioxidants; all values after the final step, PLE_{140}–W were the lowest and decreased 10.6, 6.5 and 5–fold in case of TPC, TEAC and ORAC assays, respectively. The correlation between TPC and radical scavenging values in case of QUENCHER assay was even higher than in case of extracts: TPC vs. TEAC, R^2=0.92 and TPC vs. ORAC R^2=0.94. Comparing QUENCHER results with those obtained by analyzing the extracts some interesting observations can be noticed. The sum of TPC, TEAC and ORAC values of extracts isolated by serial extraction and calculated for 1 g of DWP was quite comparable to the relevant values measured for the whole botanical material before extraction by a QUENCHER assay. For instance, TPC of BWF in QUENCHER assay before extraction was 250 mg GAE/g DWP, while the sum of extracts and residues after series PLE_{70} was 179.6 mg GAE/g DWP (Σ_{TPC} = 36.5+54.0+11.1+78.0) and after SFE–CO_2–PLE 219.1 mg GAE/g DWP (Σ_{TPC} =38.7+60.2+25.2+95). To the best of our knowledge no results have ever been published on evaluating BWF using QUENCHER method. In general, application of high pressure consecutive extraction and comprehensive evaluation of botanicals or other plant materials previously has been applied for botanicals very scarcely (Kemzūraitė et al., 2014; Kraujalis et al., 2013; Kryževičiūtė et al., 2016; Šulniūtė et al., 2016).

4.2.4. Quantification of buckwheat flower's phytochemicals

Phytochemical studies of extracts were performed by UPLC–ESI–QTOF–MS; seven constituents were identified and quantified in the analysed BWF fractions. Their concentrations in different extracts and plant material are provided in **Table 4.7**, selected chromatogram in **Fig. 4.5**, and the structures in **Fig. 4.6**. Phenolic acids and flavonoids were reported previously in different anatomical parts of buckwheat. For instance, chlorogenic acid, orientin, isoorientin, vitexin, isovitexin, rutin, quercetin, catechin and epicatechin were determined in sprouts (Aleksenko, 2013; Kim et al, 2014; Liu et al., 2008), phenolic acids in the inflorescences (Sytar, 2014), flavonoids in stems, leaves, flowers and seeds (Fabjan et al., 2003; Hinneburg&Neubert, 2005; Suzuki et al., 2009; Zielinska et al., 2012). However, to the best of our knowledge citric acid, quercetin arabinozide, quercetin galactoside, isoorientin and myricitrin have not been reported in buckwheat inflorescences previously. Some minor peaks in the chromatograms remain unknown because exact mass data obtained by QTOF–MS was not sufficient for their identification; however, phototoxic fagopyrin (MW=670.71 g/mol) was not detected.

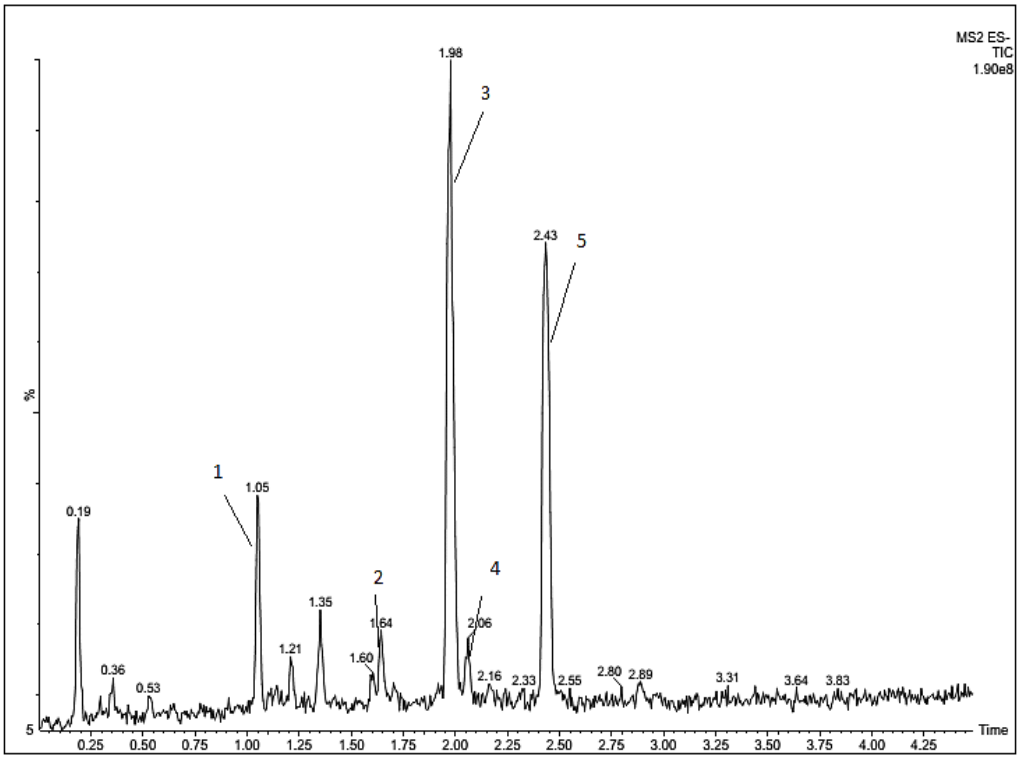


Fig. 4.5. Major phenolic compounds of PLE_{70}–ET/W extract of *F. esculentum* flowers. – chlorogenic a., 2 – isoorientin, 3 – rutin, 4 – quercetin galactoside, 5 – quercitrin

It may be clearly observed that rutin constituting 35.38–56.8 mg/g DWP is the major quantitatively constituent in non–lipophilic BWF extracts, which agrees with previously reported data for common buckwheat flowers, e.g., Dadáková and Kalinová (2010) Uddin et al. (2013) and Zielinska et al. (2012) reported 30 mg/g DWP, 31.58–56.10 mg/g DWP and 7.285–7.761 %, respectively. Acar et al. (2011) determined remarkably lower content of rutin in BWF from Turkey, 2.95 mg/g DWP. The amount of accumulated rutin and other phytochemicals may depend on various factors, such as plant cultivar, harvesting time, climatic conditions, etc.

Table 4.7. Concentration of major phenolic compounds in *F. esculentum* extracts (DWE) and plant material (DWP), in mg/g

Extraction method	Solvent	Chlorogenic acid		Citric acid		Quercetin arabinopyranozide		Quercetin galactoside	
		DWE	DWP	DWE	DWP	DWE	DWP	DWE	DWP
PLE_{70}	HX	0.21±0.00	0.01	0.31±0.00	0.01	0.11±0.00	<0.01	–	–
	AC	30.1±0.00	3.55	31.3±0.01	3.69	0.30±0.01	0.04	11.0±0.01	1.30
	ET/W	23.1±0.01	4.27	30.7±0.00	5.68	0.28±0.00	0.05	10.9±0.02	2.02
	W	23.0±0.01	1.04	29.7±0.00	1.35	0.12±0.00	0.01	–	–
	Total		**8.87**		**10.72**		**0.1**		**3.32**
PLE_{140}	HX	0.32±0.00	0.01	–	–	0.12±0.01	<0.01	–	–
	AC	21.1±0.00	3.99	19.8±0.00	3.74	0.42±0.00	0.08	11.0±0.00	2.08
	ET/W	20.2±0.00	5.62	19.4±0.00	5.39	0.33±0.00	0.09	–	–
	W	20.1±0.00	2.85	19.2±0.00	2.73	0.15±0.01	0.02	–	–
	Total		**12.47**		**11.86**		**0.19**		**2.08**
$SFE–CO_2$	CO_2	0.17±0.00	<0.01	–	–	0.11±0.00	<0.01	–	–
	CO_2–ET*	0.65±0.00	0.02	0.81±0.00	0.02	0.12±0.00	<0.01	–	–
$SFE–CO_2/PLE_{70}$	AC	30.0±0.00	3.72	31.1±0.00	3.92	0.34±0.01	0.04	10.9±0.01	1.35
	ET/W	20.4±0.01	4.63	22.7±0.00	5.15	0.22±0.01	0.05	–	–
$SFE–CO_2/PLE_{140}$	W	–	–	0.44±0.00	0.06	0.13±0.01	0.02	–	–
	Total		**8.35**		**9.13**		**0.11**		**1.35**

Table 4.7. (continued)

Quercitrin		Isoorientin		Myricitrin		Rutin	
DWE	DWP	DWE	DWP	DWE	DWP	DWE	DWP
–	–	0.08±0.00	<0.01	0.09±0.01	<0.01	–	–
76.0±0.39	8.97	–	–	–	–	100±0.11	11.8
58.6±1.52	10.8	–	–	–	–	110±0.12	20.4
55.8±0.36	2.53	–	–	–	–	70.1±0.09	3.18
	22.3		**<0.01**		**<0.01**		**35.38**
–	–	0.07±0.00	<0.01	0.08±0.00	<0.01	–	–
70.8±0.12	13.4	–	–	–	–	100±0.10	18.9
51.0±1.23	14.2	–	–	–	–	90.2±0.07	25.1
35.4±0.62	5.03	0.91±0.00	0.26	–	–	90.3±0.10	12.8
	32.63		**0.13**		**<0.01**		**56.8**
–	–	0.06±0.01	<0.01	0.08±0.00	<0.01	–	–
6.41±0.09	0.17	0.07±0.00	<0.01	0.11±0.00	<0.01.	–	–
88.0±2.46	10.9	–	–	–	–	100±0.14	12.4
26.4±0.84	5.99	–	–	–	–	101±0.12	22.9
–	–	0.07±0.01	0.01	0.11±0.00	0.02	87.2±0.04	12.6
	16.89		**0.01**		**0.02**		**47.9**

Values represented as mean ± standard deviation (n=3);
DW–dry weight; HX–hexane; AC–acetone; ET/W–ethanol/water (80/20); W–water; CO_2–ET–carbon dioxide with co–solvent ethanol.
*SFE–CO_2 with co–solvent ethanol was performed from the whole plant material and is not included in to the total content.

For instance, Kalinova et al. (2006) reported that the content of rutin in the leaves of three *F. esculentum* varieties in the period of branching and before harvest were in the range of 37.3–98.2 mg/g DWP; while during flowering period its content in flowers of Emka variety was 41.69 and 52.18 mg/g DWP in 2004 and 2005, respectively. Rutin contributed about 50% of the antioxidant capacity of flowers from common and tartary buckwheat (Zielińska et al., 2012). Rutin is known as a potent antioxidant and as the major *F. esculentum* flavonoid may be responsible for the attributed to buckwheat health benefits, including plasma cholesterol level reduction, neuroprotection, anticancer, anti–inflammatory, antidiabetic effects, and improvement of hypertension conditions (Gimenez–Bastida & Zielinski, 2015). Second quantitatively important constituent was quercitrin (quercetin–3–rhamnoside), its concentration was up to 88 mg/g DWE (SFE–CO_2–PLE_{70}–AC). It agrees with Zielińska et al. (2012); however, the latter study showed that quercitrin concentration in BWF was highly dependent on plant vegetation phase: at early flowering and seed formation state it was 0.54 and 1.8 % DWP, respectively. Total content of quercitrin isolated during consecutive extractions in our study was 1.83–4.2 % DWP. Quercitrin, as a compound demonstrating protecting effects against oxidative stress–induced injury in lung fibroblast cells as well as $DPPH^{\bullet}$, superoxide, hydroxyl radicals and intracellular ROS scavenging effects, was suggested as a biopreservative in food applications to prevent lipid peroxidation or as a health supplement to alleviate oxidative stress (Ham et al., 2012).

Particularly high concentrations of rutin were determined in ET/W and AC extracts, except for PLE_{140}–ET/W; however the latter extraction process, due to a very high extract yield, was more efficient for the most exhaustive isolation of rutin and other major polyphenolics from DWP. For instance, PLE_{140}–ET/W gave 45.0 %, 45.4 %, 47.4 %, 43.5 %, and 44.2 %, of the total chlorogenic acid, citric acid, quercetin arabinopyranoside, quercitrin and rutin from 1 g of DWP, respectively.

Regarding extraction temperature, it may be also observed that the efficiency of isolation of the quantified constituents from BWF was higher in PLE_{140}; the total content of extracted chlorogenic acid, citric acid, quercitrin and rutin was higher by 40.5, 10.6, 46.3 and 60.5 %, respectively, than in PLE_{70}. In total, PLE_{70}, PLE_{140} and SFE–CO_2–PLE gave 80.69, 116.16 and 83.76 mg/g DWP of the quantified phytochemicals. It indicates that short extraction time at high temperature is the most effective extraction process, which does not induce chemical changes. The minor quantitatively constituents were myricitrin (0.08–0.11 mg/g DWE) and isoorientin (0.06–0.91 mg/g DWE). It is interesting noting that aglycone quercetin has not been detected in the extracts analysed in our study, while small amount of this compound (29.33 µg/g) was reported in BWF from Turkey (Acar et al., 2011); however, in this case the extracts were prepared during 24 h with 90 % methanol, while in PLE extraction it was completed in 15 min. It may be suggested that longer

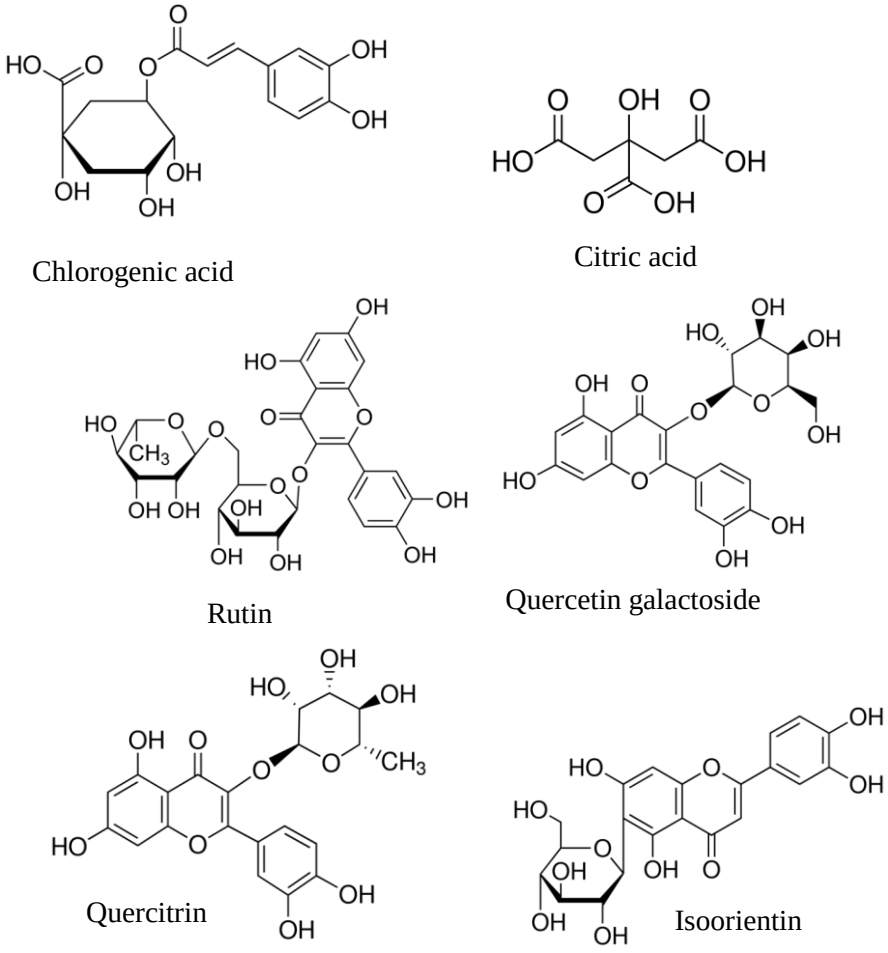


Fig. 4.6. Major compounds found in buckwheat (*Fagopyrum esculentum*) flower extracts

extraction time results in some hydrolysis of quercetin glycosides; however this assumption is not supported by the work of Zielińska et al. (2012), who found up to 0.25 % of quercetin in BWF extracts isolated with 80 % methanol by vortexing, sonicating (both 30 s) and centrifugating 5 min (5 times). Uddin et al. (2013) also determined 180–320 μg/g of quercetin in BWF extracts isolated with methanol during 0.5 h at 60 °C. In general, these findings show that the major constituents of BWF are quite stable at high temperature, which is important in selecting a proper extraction/fractionation procedure.

4.2.5. Extraction and determination of tocopherols.

The vitamin E family includes four tocopherols and four tocotrienols (α, β, γ, δ), which differ in their methyl substitutions and saturation. The HPLC analysis for

tocopherols separation was applied by using reversed–phase, which is based on the structure of side chain and the number of methyl substituent's. The linear relationship between peak area and various concentrations of tocopherols were evaluated and calibration curves demonstrated good linear relationships: α–T, R^2 = 0.999; γ–T, R^2 = 0.999; δ–T, R^2 = 0.999. The total concentration of tocopherols in the extracts isolated by PLE_{70}, PLE_{140}, SFE–CO_2 and SFE–CO_2/ET was 1381 μg/g DWE (**Fig. 4.7**). The major quantitative tocopherol was α–T (260–296 μg/g extract) and it agrees with Kalinova et al. (2006) who reported 173 and 62 μg/g DWP of α–T in BWF collected in 2004 and 2005, respectively. The highest quantity of tocopherols was found in SFE–CO_2 extracts. SFE–CO_2 modified with 10% of ethanol gave the extracts with lower concentrations of tocopherols by approx. 1.3–1.5 times than in case of pure CO_2, most likely due to dilution of tocopherols with other less lipophilic substances (co–solvent ET increased extract yield 1.48 times).

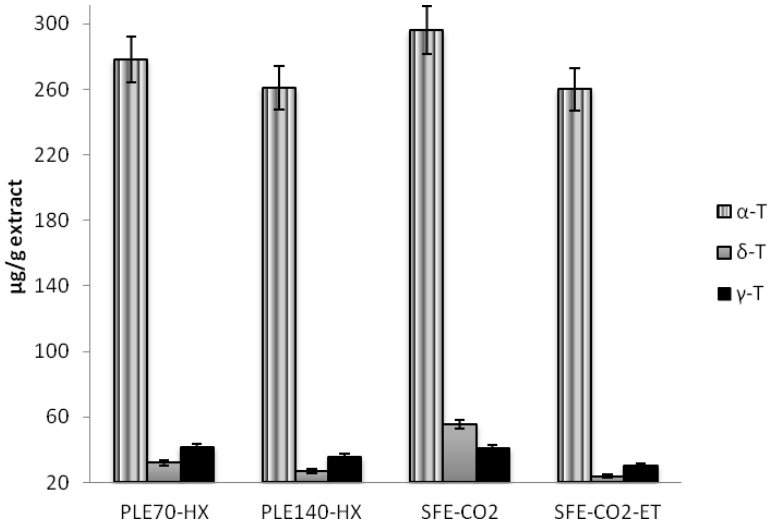


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Fig. 4.7. Concentration of tocopherols (μg/g extract) in lipophilic buckwheat flower extracts. HX–hexane; CO_2 – carbon dioxide; CO_2–ET – carbon dioxide and co–solvent ethanol. Values represented as mean ± standard deviation (n=3).

4.2.6. Effect of buckwheat flowers extracts on rapeseed oil and emulsions oxidation

Due to the increasing demand of natural antioxidants, which might be used as food additives, BWF extracts were tested in edible oil systems. Lipid oxidation is a complex phenomenon induced by various factors such as oxygen, initiators, catalysts, heat and UV light. Moreover, primary and secondary oxidation products are formed in the course of oil oxidation. Therefore, two methods were applied for evaluating antioxidative effects of BWF extracts. Oxipres method is based on evaluating oxidation induction period which can be quite precisely measured by the change of oxygen pressure in the reaction vessel due to an increase rate of hydroperoxide formation, while Rancimat method measures secondary oxidation

products. So far as both lipophilic (tocopherols) and higher polarity (hydrophilic) antioxidants (flavonoids, phenolic acids) were found in BWF extracts they were tested in pure oil (RO) and o/w (70/30) emulsion (EM).

Table 4.9. Antioxidative characteristics of buckwheat flowers extracts in rapeseed oil (RO) and emulsion (E) at 120 °C

	Oxipres at 120 °C				Rancimat at 120 °C	
Extracts additives	RO		EM		EM	
	IP	PF	IP	PF	IP	PF
RO (Control)	2.18±0.01	1	–	–	–	–
E (Control)	–		2.64	1	9.24	1
PLE_{70} HX	2.62±0.01	1.20	–	–	–	–
$SFE–CO_2$	2.74±0.02	1.26	–	–	–	–
PLE_{70}AC	–	–	3.95±0.02	1.50	11.3±0.04	1.23
PLE_{70}–ET/W	–	–	6.15±0.09	2.34	18.0±0.03	1.95
PLE_{140}–W	–	–	3.89±0.01	1.47	10.9±0.01	1.18

HX–hexane; CO_2–carbon dioxide; AC–acetone; ET/W–ethanol/water (80/20); W–water;
Values represented as mean ± standard deviation (n=3);
IP – induction period; PF – protector factor.

The oxidative stability of RO and E with BWF extracts was expressed by the autoxidation induction period (IP) and protection factor (PF) (**Table 4.9**). In general, all BWF extracts demonstrated oil and emulsion stabilizing for oxidation effects: PF varied from 1.20 to 2.34. The highest antioxidative effect against lipid oxidation demonstrated ET/W extract (PF 2.34) and it agrees with its strong antioxidant capacity (**Table 4.6**).

Antioxidant activity of buckwheat leaf's methanolic extracts was previously tested using Schaal oven and Oxipres methods. Holasova et al. (2001; 2002) reported that PF (Oxipres method) increased with increasing leaf extract addition; the value found for 15 % leaf's methanolic extract addition amounted to 1.6, while using Schaal oven test PF was 8.0 at the applied concentration of 20 %.

4.2.7. Conclusions

It is demonstrated that consecutive fractionation by using supercritical fluid and pressurized liquid extraction with increasing polarity solvents may be an effective process for biorefining buckwheat *(Fagopyrum esculentum)* flowers into valuable products, possessing strong antioxidant capacity and high concentration of health beneficial compounds, particularly rutin and quercitrin. Up to 64.05 % of extracts may be obtained from dry buckwheat flower's weight reducing the amount of solid residues down to only 13.5 %.

4.3. Biorefining of Goldenrod (*Solidago Virgaurea* L.) leaf by supercritical fluid and pressurized liquid extraction and evaluation of antioxidant properties and main phytochemicals in fractions and plant material

4.3.1. The yields of fractions isolated from *S. Virgaurea* leaf by different solvents and methods.

The reports on consecutive application of supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE) are very scarce to botanical species. In present work PLE using different polarity solvents (hexane, acetone, ethanol/water (80/20) and water) and SFE adding ethanol as co–solvent was applied for extraction of active compounds from *S. virgaurea* leaf. To the best of our knowledge, such approach has not been applied previously to goldenrod.

The yields (**Table 4.10**) of GRL lipophilic fraction ranged from 2.40 % DWP (SFE–CO_2) to 5.89 % DWP (PLE_{140}–HX). Application of 10% co–solvent ethanol increased the yield in SFE–CO_2/ET up to 2.3 times (5.52 %). The increasing polarity solvents, AC, ET/W and W gave remarkably higher yields, which were highly dependent on the temperature in PLE. Thus, the yields of AC, ET/W and W extracts at 140 °C were by 48, 67 and 17 %, respectively higher than at 70 °C. The total extract yield in PLE_{140} was by 49 % high then in PLE_{70}. For comparison, Goulart et al., (2007) reported that obtained yield from GRL after 10 min of hot water extraction was 10%; Leitner et al., (2012) reported that hot water extract yields from GR plant, varied between 3.2 and 5.6 %, i.e. many times lower than in our study. Extraction scheme involving SFE–CO_2 was slightly different and produced 55.10 % of the total extract. AC extract yield obtained by PLE_{70} from the SFE–CO_2 residue was similar as in purely PLE, while ET/W and W yields at the same temperature was by 39 % and 34% higher, respectively.

4.3.2. Antioxidative properties of *S. virgaurea* leaf using traditional procedure

Numerous plant origin materials are a good source of dietary antioxidants, which act as reducing agents, hydrogen donators and free radical scavengers. Goldenrod leaf (GRL) extracts isolated with organic solvents and water were also shown to possess strong RSC (Apáti et al., 2003, Demir et al., 2009 and Deng et al., 2015).

The results of determination of GRL antioxidant activity are summarised in **Table 4.10** Comparing the extracts it may be observed that the temperature variation (PLE_{70} vs PLE_{140}) decreased RSC of $ABTS^{\bullet+}$, ORAC and TPC of GRL from 1.02 (TPC – PLE ET/W) to 2.20 (ORAC – PLE W) times; however, due to remarkably higher yields the sum of TPC and ABTS isolated from plant material was higher in case of PLE_{140} by 37 % and 31 %, respectively. TPC values varied from 98 (SFE–CO_2/PLE_{140} W) to 185 (PLE_{70} AC) mg GAE/g extract ($p<0.05$); there were found not significant difference between PLE_{70}–AC and PLE_{140}–ET/W ($p>0.05$). In $ABTS^{\bullet+}$ and ORAC assays the highest RSC demonstrated PLE_{70}–AC, ET/W; PLE_{140}–AC, ET/W and SFE–CO2/PLE_{70} AC and ET/W extracts ($p<0.05$).

A strong correlation between TPC and $ABTS^{\bullet+}$ antioxidant activity was observed: TPC vs. $ABTS^{\bullet+}$, R^2=0.91 and TPC vs. ORAC R^2=0.60 These findings reveal the complexity of the composition of antioxidatively active constituents in GRL. Since TPC via $ABTS^{\bullet+}$ showed high correlation it could be mentioned that total phenolic content using Folin–Ciocalteau's reagent is a good predictor of the in vitro antioxidant activity for GRL extracts. Antioxidant properties of goldenrod leaf were studied previously by using different methods; for instance, Apáti et al., (2003) revealed that aqueous ethanolic extracts of goldenrod herb are good radical scavengers. The characteristic of $DPPH^{\bullet}$ scavenging activity was expressed as I_{50} that is the concentration of the sample that results in a 50% decrease of colour intensity of $DPPH^{\bullet}$ solution and it varied from 26.72 to 73.52 mg/100 ml. Demir et al., (2009) found that $DPPH^{\bullet}$ scavenging activities of the methanol and hot water extracts were concentration–dependent. At a concentration of 25 µg/ml dried methanol or water extract the scavenging activities were 12.27 and 4.72% respectively, while at a concentration of 100 µg/ml the respective activities were 30.67 and 64.26%. Ahn et al., (2015) reported that $DPPH^{\bullet}$ scavenging capacity of GR herb 75% ethanolic extract was 38.4%. Deng et al. (2015) reported that TPC, $DPPH^{\bullet}$ scavenging and ORAC values in GRL varied mostly depending on vegetative stage and extraction method. TPC, $DPPH^{\bullet}$ scavenging and ORAC values varied between 1.172 and 3.808 mg AAE/g, 0.151 and 0.547 mg AAE/g and between 17.229 and 57.855 mmol TE/g DWP, respectively. It is many times lower compared to the sum of TPC (90.9 mg GAE/g DWP) and ORAC (1282.3 µmol/g DWP) obtained in our study. GR leaf and stem powder demonstrated antioxidant activity in raw ground pork, decreasing conjugated diene, free fatty acids and tiobarbituric acid–reactive substance values (Kim et al., 2013).

Table 4.10. The yields % (w/w) and antioxidant capacity characteristics of *S. virgaurea* leaf extracts isolated by different solvents; TEAC (ABTS) and ORAC values are expressed in μmol TE/g, TPC in mg GAE/g in extract (E) and plant residues (DWR) and plant material (DWP) dry weight (DW).

Sample	Solvent	Yields		TPC			TEAC (ABTS)			ORAC		
		DWR	DWP	DWE	DWR	DWP	DWE	DWR	DWP	DWE	DWR	DWP
PLE_{70}	HX	3.86 ± 0.12^{aB}	3.86^{aB}							677 ± 13.1^{a}	26.1^{a}	26.1^{a}
	AC	12.2 ± 0.47^{c}	11.7^{c}	185 ± 10.7^{c}	22.6^{a}	21.6^{b}	1293 ± 101^{b}	158^{a}	151^{b}	2497 ± 111^{e}	305^{bc}	292^{c}
	ET/W	20.6 ± 1.15^{e}	17.3^{e}	177 ± 9.81^{c}	36.5^{c}	30.6^{c}	1338 ± 98.1^{b}	276^{c}	231^{c}	2814 ± 110^{f}	580^{de}	487^{e}
	W	15.8 ± 0.68^{d}	10.0^{c}	142 ± 5.70^{b}	22.4^{a}	14.2^{a}	1098 ± 57.7^{ab}	173^{a}	110^{a}	1985 ± 95.7^{d}	314^{b}	199^{b}
	Σ	**52.46**	**42.87**		**81.5**	**66.4**		**607**	**492**		**1225.1**	**1004.1**
PLE_{140}	HX	5.89 ± 0.00^{bC}	5.89^{bC}	–	–	–	–	–	–	540 ± 28.7^{a}	31.8^{a}	31.8^{a}
	AC	18.4 ± 1.43^{e}	17.3^{e}	163 ± 9.10^{bc}	30.0^{b}	28.2^{c}	1200 ± 67.9^{b}	221^{b}	208^{c}	1939 ± 91.4^{d}	357^{bc}	335^{c}
	ET/W	38.2 ± 0.02^{h}	28.9^{g}	174 ± 8.71^{c}	66.5^{d}	50.3^{e}	1153 ± 94.5^{b}	440^{e}	333^{e}	1579 ± 89.1^{c}	603^{e}	456^{e}
	W	31.2 ± 0.87^{g}	11.7^{c}	106 ± 7.40^{a}	33.1^{bc}	12.4^{a}	870 ± 41.7^{a}	271^{c}	102^{a}	904 ± 61.8^{b}	282^{b}	106^{ab}
	Σ	**93.69**	**63.79**		**129.6**	**90.9**		**932**	**643**		**1273.8**	**928.8**
SFE	CO_2	2.40 ± 0.08^{aA}	2.40^{aA}	–	–	–	–	–	–	511 ± 25.4^{a}	12.3^{a}	12.3^{a}
SFE	CO_2/ET*	5.52 ± 0.18^{abC}	5.52^{abC}	–	–	–	–	–	–	804 ± 39.7^{ab}	44.4^{a}	44.4^{ab}
$SFE–CO_2/PLE_{70}$	AC	13.2 ± 0.13^{c}	12.9^{cd}	169 ± 10.1^{bc}	22.3^{a}	21.8^{b}	1172 ± 84.5^{b}	155^{a}	151^{b}	2807 ± 100^{f}	371^{bcd}	362^{c}
	ET/W	28.6 ± 0.62^{f}	24.1^{f}	142 ± 9.00^{b}	40.6^{c}	34.2^{cd}	1169 ± 44.7^{b}	334^{d}	282^{d}	2583 ± 110^{ef}	739^{f}	623^{f}
$SFE–CO_2/PLE_{140}$	W	28.1 ± 0.71^{f}	15.7^{e}	98.0 ± 5.47^{a}	27.5^{ab}	15.4^{a}	950 ± 31.3^{ab}	267^{c}	149^{b}	1815 ± 92.2^{cd}	510^{cd}	285^{c}
	Σ	**74.7**	**55.10**		**90.4**	**71.4**		**756**	**582**		**1632.3**	**1282.3**

Values represented as mean ± standard deviation (n=3); different lowercase superscript letters within the same column indicate significant differences at $p<0.05$; different uppercase superscript letters within the same column indicate significant differences between lipophilic extracts at $p<0.05$.
HX–hexane; AC–acetone; ET/W–ethanol/water (80/20); W–water; CO2–ET–carbon dioxide with co–solvent ethanol.
*SFE–CO2 with co–solvent ethanol was performed separately from the whole plant material and is not included in to the total content plant material and is not included in to the total content.
DWE – dry weight extract; DWR – dry weight extraction residue; DWP – dry weight initial plant material.

4.3.3. Direct Evaluation of Antioxidant Capacity using QUENCHER procedure.

Serpen et al., (2007) developed the QUENCHER method, which determines the antioxidant activity of the whole plant material. Some insoluble antioxidatively active constituents may be strongly bound to other components in plant material matrix and are not extracted by organic solvents or enzymatic treatments without altering their molecular nature. In our case it was important to follow the changes of antioxidant indicators of plant material in the course of consecutive extractions (**Table 4.11**). Comparing TPC, $ABTS^{\bullet+}$ and ORAC values it can be noticed, that application of a non–polar solvent did not have any significant effect ($p>0.05$) on the antioxidant activity values of plant material. Significant difference was found comparing BE with other samples – PLE_{70}–W and SFE–CO_2–PLE_{140}–W ($p<0.05$). A strong correlation between TPC and $ABTS^{\bullet+}$ antioxidant activity was observed: TPC vs. $ABTS^{\bullet+}$, $R^2=0.87$ and TPC vs. ORAC $R^2=0.91$. Comparing QUENCHER results with those obtained by analyzing the extracts some interesting observations can be noticed. The sum of RSC of GRL obtained in TPC, $ABTS^{\bullet+}$ and ORAC assays by analyzing the extracts isolated by serial extraction and calculated for 1 g of DW was very similar to material before extraction values obtained by a QUENCHER assay

Table 4.11. Antioxidant characteristics of solid substances of goldenrod leaf measured by QUENCHER method; TPC expressed as mg GAE, TEAC and ORAC μmol TE in 1 g DWP.

Plant material	Solvent	TPC	ABTS	ORAC
Before extraction	–	254 ± 31.7^{b}	1429 ± 140^{c}	1417 ± 121^{c}
After PLE_{70}	HX	259 ± 31.8^{b}	1405 ± 108^{c}	1420 ± 138^{c}
	AC	250 ± 26.0^{b}	1003 ± 100^{b}	1112 ± 100^{bc}
	ET/W	190 ± 19.7^{ab}	851 ± 84.1^{ab}	900 ± 89.1^{ab}
	W	174.0 ± 15.2^{a}	672 ± 60.4^{a}	597 ± 60.7^{a}
After PLE_{140}	HX	273 ± 29.7^{b}	1391 ± 130^{c}	1531 ± 130^{c}
	AC	244 ± 24.1^{b}	991 ± 84.8^{b}	1247 ± 111^{bc}
	ET/W	203 ± 20.0^{ab}	697 ± 70.2^{ab}	1000 ± 101^{b}
	W	193 ± 12.7^{ab}	515 ± 50.9^{a}	737 ± 61.3^{a}
After SFE	CO_2	226 ± 19.8^{b}	1472 ± 120^{c}	1503 ± 117^{c}
After SFE–CO_2/PLE_{140}	W	158 ± 13.1^{a}	567 ± 59.4^{a}	593 ± 50.2^{a}
After SFE–CO_2/ET	CO_2/ET	191 ± 14.1^{ab}	1215 ± 10^{bc}	1414 ± 130^{c}

Values represented as mean ± standard deviation (n=3); different superscript letters within the same indicate significant differences. (GraphPad Prism 5. Tukey's Least Significant difference (LSD), $p<0.05$).

HX–hexane; AC–acetone; ET/W–ethanol/water(80/20); W–water; CO_2–ET–carbon dioxide with co – solvent ethanol.

For instance, GRL TPC QUENCHER value before extraction was 254 mg GAE/g DWP and sum of extracts and residues after PLE_{70} series extraction was 240.4 mg GAE/g DW (Σ_{TPC} (174+14.2+30.6+21.6)=240.4 mg GAE/g DW). Several

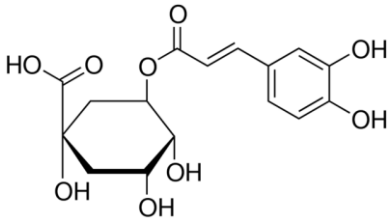
reasons may be raised to explain these findings. First of all, using in series extraction majority of active compounds was taken out in previous steps of extraction. Another reason as it was already mentioned, some part of antioxidatively active compounds may remain in the matrix after extraction because they are bound to other constituent. Finally, traditional and QUENCHER procedures reveal the full antioxidative activity in extracts and residues. To the best of our knowledge no results have ever been published on evaluating goldenrod leaf using QUENCHER method. In general, application of high pressure consecutive extraction and comprehensive evaluation of botanicals or other plant materials previously has been applied for botanicals very scarcely (Kraujalis, et al., 2013; Kemzūraitė et al., 2014; Kryževičiūtė et al., 2015, Šulniūtė et al., 2016).

4.3.4. Preliminary Characterization of Goldenrod Leaf (*Solidago virgaurea* L.) Phytochemicals by Chromatographic Analysis.

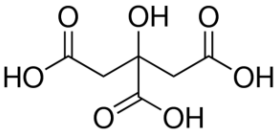
Various poly(phenols) were identified in golden rod leaf and herb previosely: chlorogenic, rosmarinic, cafeic acids, afzelin, astragalin, hyperoside, isoquercitrin, leiocarposide, nikotiflorin, quercitrin, quercetin, rutin, (Bader et al., 1998, Apáti et al., 2002, 2003 Roslon et al., 2014, Radušienė et al., 2015).

The preliminary phytochemical screening of extracts in our study was performed by UPLC–ESI–QTOF–MS; seven constituents were identified and quantified in the analysed GRL fractions. Their concentrations in different extracts and plant material are provided in **Table 4.12,** selected chromatogram in **Fig. 4.9**, and the structures in **Fig. 4.8**. It may be clearly observed that quercitrin is the major quantitatively constituent in GRL extracts (except nonpolar extracts). Radušienė et al., (2015) reported that quercitrin quantity in aqueous/methanol leaf's extract varied between 3.49–32.87mg/g DWP depending on goldenrod species, i.e. 1.3–12.6 times lower than in our study, where total quercitrin quantity was 43.96 mg/g DWP (SFE–CO_2/PLE_{140}). Particularly high concentrations of quercitrin were determined in PLE_{140}–ET/W (76.0 mg/g DWE), PLE_{140}–AC (80.9 mg/g DWE) and PLE_{70}–AC (84.6 mg/g DWE). PLE_{140}ET/W due to a very high extract yield was the most efficient solvent in the extraction of quercitrin and other major polyphenolics from GRL (except quercetin quantity, where PLE_{140}–AC gave 20.9% more than PLE_{140}–ET/W). For instance, extraction step with PLE_{140}–ET/W gave 69%, 41%, 52%, 52 %, 39%, 50 and 54%, of the total chlorogenic a., citric a., quercetin arabinopyranozide, hyperoside, quercetin, quercitrin and myricitrin, respectively. It may be also observed that the efficiency of isolation of the quantified constituents from GRL was higher in PLE_{140}; the total content of extracted chlorogenic a., citric a., avicularin, hyperoside, quercetin, quercitrin, myricitrin and rutin was higher by 1.5, 1.5, , 1.6, 1.6, 3.1, 1.6, 2.0 and 8.9 times, respectively, comparing to PLE_{70}.

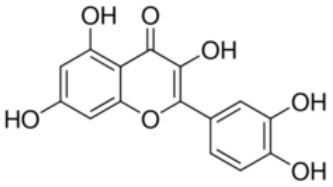
Second quantitatively important constituent was hyperoside (Quercetin 3–D–galactoside), its concentration was 17.2–28.11mg/g DWP. Radušienė et al., (2015) reported that in aqueous/methanol leaf's extract hyperoside quantity was 0.27–2.60 mg/g DWP.



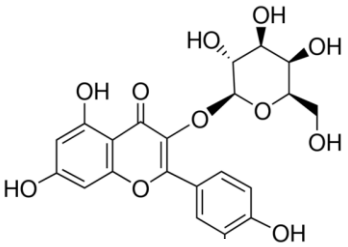
Chlorogenic acid



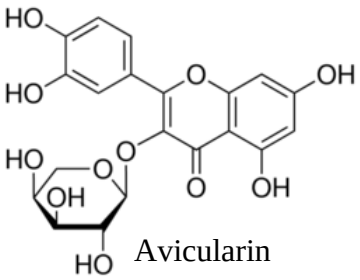
Citric acid



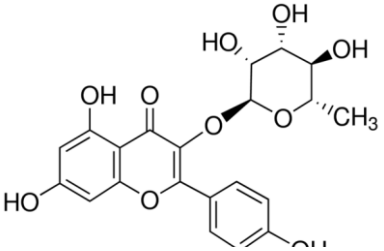
Quercetin



Hyperoside



Avicularin



Quercitrin

Fig. 4.8. Major compounds found in Goldenrod leaf extracts

Roslon et al., (2014) found that in acetone/ethyl acetate GR herb extracts hyperoside quantity varied between 0.18–0.84 mg/g DWP.A valid quantity of avicularin (Quercetin 3–O–α–L–arabinopyranoside) was found after consecutive extraction (PLE_{70} – 9.64; PLE_{140} – 15.49 and $SFE–CO_2$/PLE – 9.97 mg/g DWP). Also, compounds marked as X_1 and X_2 had the same molecular weight as avicularin (434.35 g/mol) and their quantity is expressed in avicularin units. X_1 quantity ranged from 2.02–3.34 and X_2 6.91–10.69 mg/g DWP depending on extraction temperature.

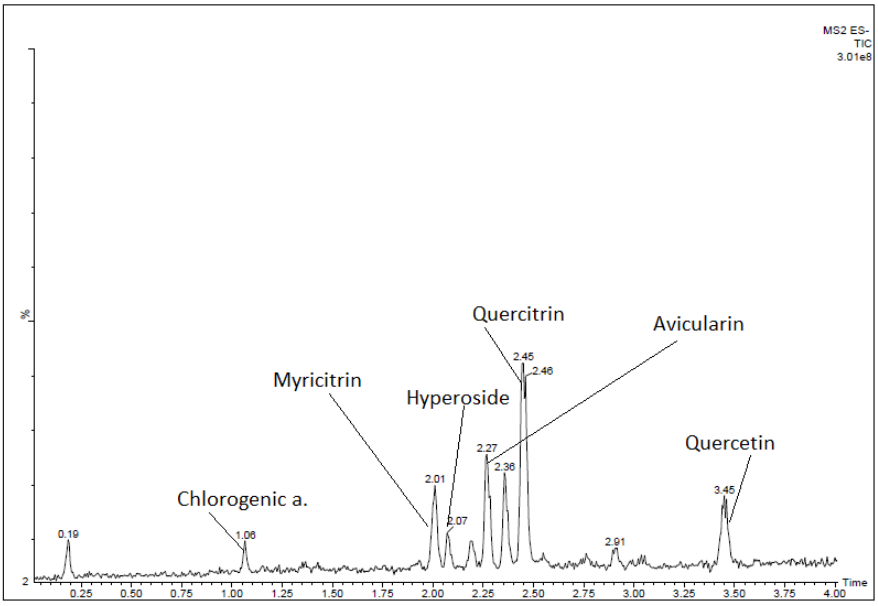


Fig. 4.9. Major phenolic compounds of Goldenrod leaf PLE_{70}–AC extract

Another important phenolic compounds present in GR herb are poly–phenolic acids. Demir et al., (2009) showed that they are in part responsible for antioxidant, anti–inflammatory, and bile flow enhancing properties of goldenrod herb. Chlorogenic and citric acids were found in our study. Chlorogenic a. quantity after in series extraction varied between 10.24–15.79 mg/g DWP, depending on extraction temperature. Radušienė et al., (2015) reported that chlorogenic a. quantity in aqueous/methanol leaf‘s extracts was 20.10–31.31 mg/g DWP. Roslon et al., (2009) reported that chlorogenic acid in aqueous GR herb extract varied between 1.59–4.41 mg/g DWP.

Table 4.12. Concentration of major phenolic compounds in *S. virgaurea* extracts (DWE) and plant material (DWP), in mg/g

Extraction method	Solvent	Hyperoside		Quercetin		Quercitrin		Myricitrin		Rutin	
	HX	DWE	DWP	DWE	DWP	DWE	DWP	DWE	DWP	DWE	DWP
	AC	–	–	–	–	–	–	–	–	–	–
	ET/W	55.4±2.29	6.48	23.8±0.64	2.78	84.6±2.98	9.90	9.41±0.37	1.10	–	–
PLE_{70}	W	42.7±0.15	7.34	8.37±0.15	1.45	67.9±0.33	11.8	8.26±0.29	1.43	3.8±0.02	0.14
	Total	33.8±0.03	3.38	–	–	62.0±0.37	6.20	3.57±0.14	0.36	–	–
	HX		**17.2**		**4.23**		**27.9**		**2.89**		**0.14**
	AC	–	–	–	–	–	–	–	–	–	–
	ET/W	50.2±0.26	8.68	35.8±0.62	6.19	80.9±3.11	14.0	10.6±0.48	1.83	1.31±0.00	0.23
PLE_{140}	W	50.5±0.86	14.6	17.7±0.94	5.12	76.0±0.68	22.0	10.5±0.55	3.03	3.54±0.00	1.02
	Total	41.3±0.81	4.83	15.0±0.11	1.76	68.0±1.21	7.96	6.84±0.41	0.80	–	–
	CO_2		**28.11**		**13.07**		**43.96**		**5.66**		**1.25**
$SFE–CO_2$	CO_2–ET**	–	–	–	–	–	–	–	–	–	–
	AC	1.04±0.00	0.06	4.78±0.10	0.26	9.93±0.45	0.55	–	–	–	–
$SFE–CO_2/PLE_{70}$	ET/W	70.1±1.85	9.04	24.4±0.17	3.15	94.1±1.61	12.1	12.8±0.46	1.65	0.69±0.00	0.09
	W	40.1±0.75	9.66	0.87±0.03	0.21	57.6±1.26	13.9	7.71±0.19	1.86	3.24±0.10	0.78
$SFE–CO_2/PLE_{140}$	**Total**	38.7±0.15	6.08	–	–	50.7±1.02	7.96	–	–	–	–
			24.78		**3.62**		**33.96**		**3.51**		**0.87**

Table 4.12. (continued)

Extraction method	Solvent	Chlorogenic acid		Citric acid		Avicularin		X_1*		X_2*	
		DWE	DWP	DWE	DWP	DWE	DWP	DWE	DWP	DWE	DWP
PLE_{70}	HX	–	–	–	–	–	–	–	–	–	–
	AC	6.29±0.39	0.74	4.67±0.14	0.55	28.0±0.80	3.28	5.88±0.15	0.69	21.8±1.19	2.55
	ET/W	39.1±1.67	6.76	30.5±1.57	5.28	25.1±0.42	4.34	5.73±0.49	0.99	17.3±0.86	2.99
	W	27.4±0.22	2.74	20.3±0.87	2.03	20.2±0.22	2.02	3.43±0.06	0.34	13.7±0.76	1.37
	Total		**10.24**		**7.86**		**9.64**		**2.02**		**6.91**
PLE_{140}	HX	–	–	–	–	–	–	–	–	–	–
	AC	14.3±0.58	2.47	9.62±1.05	1.66	28.0±0.24	4.84	6.31±0.15	1.09	21.3	3.68
	ET/W	37.6±0.36	10.9	27.7±0.45	8.01	27.6±0.25	7.98	5.99±0.08	1.73	18.1	5.23
	W	20.7±0.47	2.42	17.0±0.74	1.99	22.8±0.54	2.67	4.42±0.21	0.52	15.2	1.78
	Total		**15.79**		**19.52**		**15.49**		**3.34**		**10.69**
$SFE–CO_2$	CO_2	–	–	–	–	–	–	–	–	–	–
	CO_2–ET**	–	–	–	–	3.45±0.20	0.19	0.18±0.00	0.01	3.51±	0.19
$SFE–CO_2/PLE_{70}$	AC	11.6±0.47	1.50	7.69±0.18	0.99	36.6±0.27	4.72	9.68±0.31	1.25	28.2±0.94	3.64
	ET/W	42.5±0.60	10.2	36.6±0.97	8.82	21.7±0.41	5.23	4.50±0.23	1.08	14.6±0.61	3.52
$SFE–CO_2/PLE_{140}$	W	22.7±0.17	3.56	0.77±0.00	0.12	0.15±0.00	0.02	–	–	0.17±0.00	0.03
	Total		**15.26**		**9.93**		**9.97**		**2.33**		**7.19**

Values represented as mean ± standard deviation (n=3);
DW–dry weight; HX–hexane; AC–acetone; ET/W–ethanol/water (80/20); W–water; CO_2–ET–carbon dioxide with co–solvent ethanol.
X_1,X_2 quantity is expressed in avicularin units.
**SFE–CO_2 with co–solvent ethanol was performed from the whole plant material and is not included in to the total content.

It is known that citric acid acts as antioxidant synergist. (Akaranta & Akaho, 2012; Hras et al., 2000). In our study, citric acid quantity varied between 7.86–19.72 mg/g DWP. To the best of our knowledge citric acid was not reported previously in GRL extracts.

The minor quantitatively constituents were rutin (0.09 – 1.02mg/g E) and myricitrin (0.8 – 3.03 mg/g E). In literature, the quantity of rutin in GRL aqueous/methanol extracts varied between 0.58–17.47 mg/g DWP depending on goldenrod species. (Radušienė et al., 2015). Roslon et al., (2014) reported that quantity of rutin in acetone/ethylacetate GR herb extracts was 0.88–3.88 mg/g DWP depending on GR species. For the best of our knowledge myricitrin was not reported previously in GRL.

Phenolic ester – leiocarposide, recognized as a lead structure for the quality assurance of *Solidaginis virgaureae* herba (Bader et al., 1998), was not identified in our study. Some other studies showed that in goldenrod methanol's extract chloroform fraction leiocarposide ranged between 0.14 – 0.24 % in DWP (Thiem et al., 2001). Luck et al., (1999) reported that leiocarposide quantity varied between 0 – 1.6% DWP, mostly depending on growth area, harvesting time, cultivation origin.

In general, these findings show that the major bioactive constituents of GRL might be strongly embedded in the tough solid plant particles and they remain stable at high temperature, which is important in selecting a proper extraction/fractionation procedure.

4.3.5. Extraction and determination of tocopherols.

The vitamin E family includes four tocopherols (alpha, beta, delta, gama) and four tocotrienols (alpha, beta, delta, gama), which differ in their methyl substitutions and saturation.

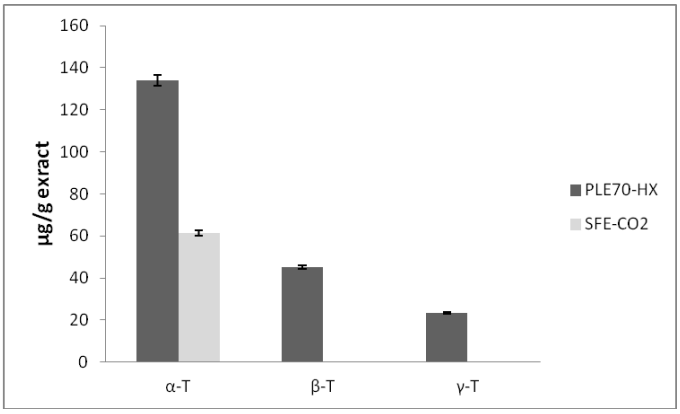


Fig. 4.10. Concentration of tocopherols (µg/g extract) in lipophilic *S. virgaurea* extracts. HX–hexane; CO2–carbon dioxide; CO2–ET–carbon dioxide co – solvent ethanol. Values represented as mean ± standard deviation (n=3);

The HPLC analysis for tocopherols separation was applied by using reversed–phase, which is based on the structure of side chain and the number of methyl substituents.

The linear relationship between peak area and various concentrations of tocopherols were evaluated and calibration curves demonstrated good linear relationships: α–T, $R^2 = 0.999$; γ–T, $R^2 = 0.999$; δ–T, $R^2 = 0.999$.

The major quantitative tocopherol was Alpha–T (61.4 – 134 μg/g extract) and it agrees with Ahn et al., 2015.The highest quantity of tocopherols was found in PLE70–HX extracts (**Fig. 4.10**), in total 202.6 μg/g extract.

4.3.6. Effect of *S. virgaurea* L. flowers extracts on rapeseed oil and emulsions oxidation

Lipid oxidation is a complex phenomenon induced by oxygen in the presence of various catalyzing factors. The change of oxygen pressure in the reaction vessel at the end of the induction period, which indicates the beginning of a rapid formation of hydroperoxides, can be quite precisely measured by using the Oxipres method. Rancimat is used as a standard technique for measuring the secondary oxidation products.

The oxidative stability of oil and emulsion with goldenrod extracts was evaluated by the autoxidation induction period (IP) and protection factor (PF) (**Table 4.13**).

Table 4.13. Antioxidative characteristics of *S.virgaurea* leaf extracts in rapeseed oil (RO) and emulsion (E) at 120 °C.

	Oxipres at 120 °C				Rancimat at 120 °C	
Extracts additives	RO		EM		EM	
	IP	PF	IP	PF	IP	PF
RO (Control)	2.18±0.01	1	–	–	–	–
E (Control)	–		2.64	1	9.24	1
PLE_{70} HX	1.07±0.03	0.49	–	–	–	–
SFE–CO_2	0.89±0.06	0.41	–	–	–	–
PLE_{70}AC	–	–	2.95±0.00	1.12	9.13±0.01	1.01
PLE_{70}–ET/W	–	–	3.86±0.12	1.46	12.9±0.00	1.40
PLE_{140}–W	–	–	3.05±0.06	1.16	10.7±0.02	1.16

HX–hexane; CO_2–carbon dioxide; AC–acetone; ET/W–ethanol/water (80/20); W–water;
Values represented as mean ± standard deviation (n=3);
IP – induction period; PF – protector factor.

In general, all extract additives demonstrated emulsion stabilizing for oxidation effects (PF was 1.12-1.46), except for HX and CO_2 extracts. It was reported that added to vegetable oils do not improve their oxidative stability because the required concentration of these endogenous to oil seeds compounds are still present even in the refined oils (Abuzaytoun & Shahidi, 2006; Hamam & Shahidi, 2006; Kiokias et al., 2008). The effect of additives on emulsion stabilization decreased in the following order: ET/W>W<AC. The highest antioxidative effect against lipid oxidation demonstrated ET/W extract (PF=1.46) and it is in agreement with antioxidative properties measured *in vitro* (**Table 4.10**).

4.3.7. Conclusion

Goldenrod (*Solidago virgaurea* L.) leaf is a good source of valuable biologically active substances possessing antioxidant and other beneficial health properties. Assessment of antioxidant properties by several in vitro assays demonstrated that in some cases temperature demonstrated significant differences and water residues after in series extraction possesses many times lower antioxidant potential than in initial plant material. Eight phenolic constituents were identified in GRL extracts.

V. CONCLUSIONS

1. The application of different processing schemes demonstrated that the total yields (sums of all extractions) from *B. crassifolia* leaves (BL) and roots (BR), *Fagopyrum Esculentum* (BWF) flowers and *S. virgaurea* leaves (GRL) were very high, and highly dependent on the extraction temperature. The highest extraction yields obtained using pressurized PLE at 140 °C temperature.

2. There were remarkable variations in the antioxidant capacity values between applied solvent and extraction temperature.

2.1. The highest antioxidant capacity demonstrated ethanol and acetone plants extracts.

2.2. The highest antioxidant capacity obtained using pressurized liquid extraction at 140 °C temperature.

2.3. All analysed plant extracts demonstrated oil and emulsion stabilizing effects.

3. Consecutive extractions with the increasing polarity solvents gradually decreased antioxidant potential of extraction residues; however, the final residues were still antioxidatively active materials.

3.1. Pressurized liquid extraction at 140 °C temperature the most efficiently decreased antioxidant capacity of BL and BR and BWF residues.

3.2. Combined supercritical fluid extraction and pressurized liquid extraction at 140 °C temperature the most efficiently decreased antioxidant capacity of GRL residues.

4.1. It may be clearly observed that bergenin was the major quantitatively constituent both in BL and BR however the total amount of extracted bergenin from the roots was remarkably higher than from the leaves. Ellagic acid was detected only in BL extracts.

4.2. Rutin was the major quantitatively constituent in non–lipophilic BWF extracts. Second quantitatively important constituent was quercitrin. The total quantity of tocopherols in lipophilic extracts was 1381 μg/g extract. The main tocopherol was α–tocopherol (260–296 μg/g extract).

4.3. Quercitrin was the major quantitatively constituent in GRL extracts. Second quantitatively important constituent was hyperoside. The main tocopherol in lipophilic extracts was α–tocopherol (61.4 – 134 μg/g extract).

SANTRAUKA

Simboliai ir santrumpos

AA – antioksidacinis aktyvumas;
AC – acetonas;
AL – augalo liekana;
BFJK – bendrasis fenolinių junginių kiekies;
BŠ – storalapės bergenijos šaknys;
DRSG – deguonies radikalo surišimo geba;
ESCH – efektyvioji skysčių chromatografija;
ET/V – etanolio vandens mišinys;
ET–etanolis;
ETAS – ekstrakcija organiniais tirpikliais aukštame slėgyje;
GRE – galo rūgšties ekvivalentai;
HX – heksanas;
IP – indukcinis periodas;
PRAM – pradinė augalo masė;
RA – rapsų aliejus;
RSG – radikalų sujungimo geba;
SK – stabilumo koeficientas;
SKE – superkrizinė ekstrakcija;
TE – trolokso ekvivalentai;
UESCH – ultra efektyvioji skysčių chromatografija;
V – vanduo;
VPAA – vaistiniai, prieskoniniai, aromatiniai augalai.

I. ĮVADAS

1.1. Darbo aktualumas

Naturalių antioksidantų bei kitų fitochemikalų paieška, jų savybių įvertinimas, išskyrimo bei panaudojimo būdai dėl daugelio priežasčių tapo viena populiariausių maisto pramonės, žemės ūkio bei mitybos temų. Svarbiausia iš jų – nenuginčijamas teigiamas junginių, išskirtų iš įvairių augalų, poveikis žmogaus sveikatai. Taip pat didelį susidomėjimą lėmė spartus jų pritaikymas funkcionaliojo maisto, maisto papildų, vaistų gamyboje, kosmetikos pramonėje, o taip pat platus dar neištirtų ar menkai tirtų augalų pasirinkimas, didelis žmonių susidomėjimas natūraliais maisto priedais, pažangių analitinių metodų, leidžiančių greitai ir efektyviai išskirti bioaktyvius junginius iš augalo matricos atsiradimas bei greitas junginių savybių charakterizavimas. Vaistiniai prieskoniniai bei aromatiniai augalai (VPAA) tradiciškai naudojami liaudies medicinoje kaip priemonė turinti raminamąjį, priešvėžinį, antidiabetinį, priešuždegiminį, imunostimuliacinį, antimikrobinį, antioksidacinį, antivirusinį, antihipertenzinį ir kitokį teigiamą poveikius. Artemisinino, kuris efektyviai sunaikina maliariją sukeliančius pirmuonis, išskyrimas iš saldžiojo kiečio (*Artemisia Annua*) ir išsamus įvertinimas yra vienas iš labiausiai įkvepiančių pastarojo dešimtmečio, natūralios medicinos, pavyzdžių (Tu, 2011).

VPAA priskiriami ekonominiu požiūriu naudingųjų augalų grupei, kadangi jie yra svarbios, įvairios paskirties funkcionaliųjų komponentų gamybos žaliavos. Iš jų išskirtos medžiagos (antioksidantai, polifenoliai, alkaloidai ir kt.) gali būti panaudojamos funkcionaliojo maisto produktų, maisto papildų, farmacijos, kosmetikos ir kitų preparatų kūrimo ir gamybos tikslais. Plačiausiai pasaulyje naudojami VPAA, pasižymintys antioksidaciniu poveikiu, priklauso kelioms augalų šeimoms: Lamiaceae (rozmarinas, šalavijas, raudonėlis, mairūnas, čiobrelis, mėta, bazilikas, melisa); Apiaceae (kuminas, kmynas, pankolis); ir Zingiberaceae (ciberžolė, imbieras) (Škrovankova et al., 2012). Įvertinant milžinišką VPAA bioįvairovę, didelė jų dalis iki šiol vis dar labai mažai tirta. Trūksta tyrimų, skirtų fitocheminių medžiagų išskyrimo būdams ir procesams optimizuoti, kurie padidintų tokios augalinės žaliavos pramoninio perdirbimo ir panaudojimo galimybes. Todėl pastaruoju metu sparčiai plėtojama kompleksinio perdirbimo koncepcija vadinama biorafinavimu (kartais agrorafinavimu), kai ieškoma būdų kaip optimizuoti tokios augalinės žaliavos perdirbimą, siekiant kuo pilniau išnaudoti visas augalo anatomines ir sudėtines dalis. Šiuo metu pramonėje ir laboratorijose plačiai tebenaudojami bei tiriami tradiciniai augaluose esančių medžiagų išskyrimo būdai – tokie, kaip ekstrahavimas vandeniu, organiniais tirpikliais ir jų mišiniais, distiliavimas vandens garais, presavimas. Per pastaruosius kelis dešimtmečius sukurti nauji augalinės žaliavos apdorojimo ir veikliųjų medžiagų išskyrimo iš įvairių matricų metodai, tokie kaip, pagreitinta ekstrakcija organiniais tirpikliais aukštame slėgyje, ekstrakcija, panaudojant įvairius pagalbinius fizikinius poveikius, pvz., mikrobangomis ir ultragarsu, ekstrakcija suskystintomis dujomis, kietafazė fermentacija ir ypač ekstrakcija superkriziniais skysčiais. Nauji ekstrakcijos ir

frakcionavimo būdai (superkriziniais skysčiais, aukšto slėgio organiniais tirpikliais ir vandeniu) atveria plačias galimybes aukštos vertės augalinės kilmės komponentų išskyrimui iš įvairių matricų ir jų frakcionavimui. Keičiant ekstrakcijos slėgį, temperatūrą, tirpiklio srauto greitį bei panaudojant tirpiklių sistemos modifikatorius poliškumui pakeisti, galima pakankamai selektyviai išekstrahuoti pageidaujamas frakcijas. Kompleksinis šių būdų panaudojimas, išskiriant iš botaninių matricų tame tarpe ir pasirinktų tyrimo objektų, vetingąsias frakcijas nebuvo taikytas iki šiol, o tai atveria galimybes efektyviai išskirti naudingas augalų medžiagas ir pagaminti jų frakcijas, labiausiai tinkamas panaudojimui ir kitų produktų kūrimui.

1.2. Darbo tikslas ir uždaviniai

Darbo tikslas: įvertinti storalapės bergenijos (*Bergenia crassifolia* L.), grikių žiedynų (*Fagopyrum esculentum* Moench.) ir europinės rykštenės (*Solidago virgaurea* L.) perdirbimo galimybes į vertingas funkcines medžiagas, taikant biorafinavimo koncepciją.

Uždaviniai:

1. Ištirti biorafinavimo, taikant ekstrakciją organiniais tirpikliais aukštame slėgyje bei ekstrakciją superkriziniu anglies dioksidu, būdus ir įvertinti biorafinavimo koncepcijos pritaikomumą, siekiant gauti didžiausią tirtų augalų frakcijų išeigą.
2. Įvertinti gautų atskirų frakcijų, naudojant skiringus tirpiklius bei ekstrakcijos metodus, antioksidacines savybes skirtingais *in vitro* bei *in situ* (saulėgrąžų aliejuje bei emulsijose (riebalai/vanduo)) metodais.
3. Įvertinti netirpių medžiagų liekanų antioksidacines savybes, po kiekvieno ekstrakcijos etapo.
4. Nustatyti atskirų frakcijų fitocheminę sudėtį, naudojant chromatografinius bei spektroskopinius metodus.

1.3. Mokslinis darbo naujumas

Didelė dalis anksčiau atliktų tyrimų, išskiriant bioaktyviąsias frakcijas iš vaistinių, prieskoninių bei aromatinių augalų, buvo atliekami taikant tradicinius ekstrakcijos metodus, naudojant organinius, dažniausiai toksiškus tirpiklius tokius, kaip acetonas, metanolis, etilo acetatas ir kt. Taip pat dauguma atliktų tyrimų buvo naudojami tik analitiniais tikslais, o praktiniam panaudojimui augalai dažniausiai buvo ekstrahuojami etanolio vandens mišiniais, naudojant daug laiko reikalaujančius maišymo bei filtravimo būdus.

Tuo tarpu aukšto slėgio metodai, tokie kaip pakopinė ekstrakcija superkriziniu anglies dvideginiu (SKE–CO_2) bei ekstrakcija organiniais tirpikliais aukštame slėgyje bei jų kombinacijos iki šiol nebuvo taikyti aktyvių junginių frakcijų išskyrimui iš VPAA.

1.4. Praktinė vertė

1. Laboratorinėmis sąlygomis išbandytos išsamios VPAA biorafinavimo schemos sudaro sąlygas pilniau išnaudoti augalų masę bei išgauti keletą vertingųjų frakcijų, pasižyminčių skirtinga fitochemine sudėtimi. Tyrimų rezultatai gali būti lengvai pritaikomi pilotinės gamybos lygmeniu, o vėliau ir pramoniniam VPAA perdirbimui.

2. Atsižvelgiant į tai, jog minėti ir šiame darbe taikyti metodai turi keletą svarbių pranašumų, tokių kaip tausojančios chemijos principų pritaikymas, nuoseklių ir sisteminių tyrimo metodų bei schemų panaudojimas VPAA, norint išgauti vertingąsias frakcijas, pasižyminčias antioksidaciniu bei kt. aktyvumu, gali būti labai svarbūs natūralių funkcinių ingredientų išgavimui bei pritaikymui įvairiose srityse.

1.5. Ginamieji teiginiai

Biorafinavimo koncepcijos pritaikymas pasirinktų tiriamųjų augalų (*Bergenia crassifolia* L., *Fagopyrum esculentum* Moench. ir *Solidago virgaurea* L.) perdirbimui taikant daugiapakopinę ekstrakciją tradiciniais bei aukšto slėgio metodais, yra efektyvus būdas išskirti vertingąsias augalo frakcijas, pasižyminčias antioksidaciniu aktyvumu bei efektyviu kai kurių fitocheminių medžiagų išgavimu.

1.6. Darbo rezultatų publikavimas

Disertacijos tema išspausdinti 3 straipsniai Clarivate Analytics pagrindinio sąrašo leidiniuose. Darbo rezultatai pristatyti 5–iose tarptautinėse konferencijose.

1.7. Disertacijos struktūra

Disertacija parašyta anglų kalba. Ją sudaro simbolių ir santrumpų sąrašas, įvadas, literatūros apžvalga, tyrimų objektai ir metodai, išvados bei literatūros sąrašas (245 šaltiniai). Disertacijos apimtis 114 puslapių, 15 lentelių ir 20 paveikslų.

II. TYRIMŲ OBJEKTAI IR METODAI

2.1. Tyrimų objektai

Tyrimams naudoti Lietuvoje, Dzūkijos nacionaliniame parke (Panaros km., Varėnos raj., Lietuva), užauginti bergenijos (*Bergenia crassifolia* L.) lapai bei šaknys (2012 metų derlius), grikių (*Fagopyrum esculentum* Moench.) žiedynai bei rykštenės (*Solidago virgaurea* L. lapai (2013 metų derlius).

Smulkinti storalapės bergenijos lapai | Smulkintos storalapė bergenijos šaknys | Smulkinti europinės rykštenė lapai | Smulkinti grikių žiedai

2.1. pav. Tyrimų objektai

2.2. Tyrimų metodai

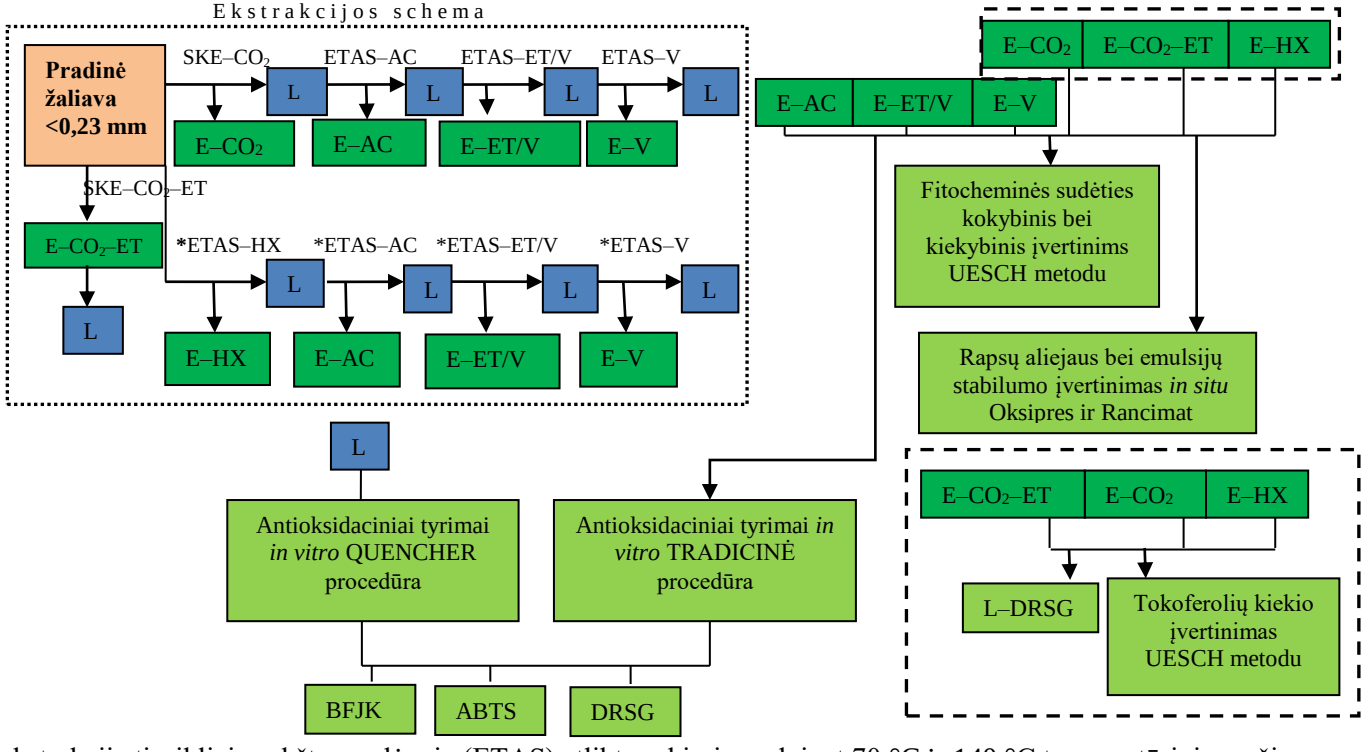
Bandiniai prieš tyrimus susmulkinti iki 0,2 mm dalelių dydžio. Taikant ekstrakciją organiniais tirpikliais aukštame slėgyje (ETAS) bei superkrizinę ekstrakciją anglies dioksidu (SKE) bei jų kombinacijas buvo siekiama išgauti kuo didesnes frakcijų išeigas, pasižyminčias antioksidaciniu aktyvumu (AA). Augalų tyrimo schema pavaizduota **2.2 pav.**

Bendras fenolinių junginių kiekis (BFJK) skystojoje frakcijoje įvertintas Folin–Ciocalteu metodu, antioksidacinis aktyvumas – naudojant 2,2'–azino–bis– (–3–etilbenztiazolin–6–sulfono rūgšties ($ABTS^{\bullet+}$) katijono radikalo blukinimo metodą bei deguonies radikalo surišimo gebą (DRSG). BFJK rezultatai išreikšti mg galo rūgšties ekvivalentų, o $ABTS^{\bullet+}$ bei DRSG µmol trolokso ekvivalentų grame ekstrakto (E) augalo liekanos (AL) bei pradinės augalo masės (PRAM).

BFJK, $ABTS^{\bullet+}$ bei DRSG sausoje pradinėje žaliavoje bei liekanoje po kiekvieno ekstrakcijos etapo įvertintas taikant QUENCHER procedūrą. BFJK rezultatai išreikšti mg galo rūgšties ekvivalentų, o $ABTS^{\bullet+}$ bei DRSG µmol trolokso ekvivalentų grame pradinės augalo masės (PRAM).

Ekstraktų fitocheminė sudėtis tirta ultraefektyviosios skysčių chromatografijos sistema su kvadrupoliniu ir skriejimo laiko masių analizatoriumi (UESCH–K–SL–MA) bei efektyviosios chromatografijos metodais.

Antiokisdacinis aktyvumas *in situ* rapsų aliejuje bei emulsijose (riebalai/vanduo), tirti naudojant oksipres ir rancimat metodus.



*– ekstrakcija tirpikliais aukštame slėgyje (ETAS) atlikta askirai naudojant 70 °C ir 140 °C temperatūrinius režimus;
E – ekstraktas; L – ekstrakcijos liekana

2.2. pav. Augalų tyrimo schema

III. DARBO REZULTATAI IR JŲ APTARIMAS

3.1. Storalapės bergenijos (*Bergenia crassifolia* L.) augalo anatominių dalių biorafinavimas, taikant aukšto slėgio ekstrakcijos metodus, antioksidacinių savybių bei pagrindinių aktyviųjų junginių įvertinimas

3.1.1. Storalapės bergenijos (*Bergenia crassifolia* L.) lapų bei šaknų ekstraktų išeigų įvertinimas

Vaistiniai, prieskoniniai bei aromatiniai augalai (VPAA) yra sudėtingos botaninės matricos, kuriose kaupiasi skirtingo poliškumo tiek tirpūs tiek netirpūs sudėtiniai komponentai. Todėl biorafinavio schemų plėtojimas leidžia išskirti frakcijas, pasižyminčias skirtingomis savybėmis, kurios galėtų būti pritaikytos įvairių produktų gamyboje. Tuo pačiu frakcijų išeigos yra vienas iš svarbiausių parametrų, siekiant procesus pritaikyti gamybos pramonėje.

Bergenijos lapų (BL) bei šaknų (BŠ) frakcionavimas taikant, nepolinius tirpiklius, tokius, kaip heksanas (HX) bei anglies dioksidas, skirtas išgauti lipofiliniams komponentams, kurie ypač svarbūs kosmetikos pramonėje. Išeigos, taikant kombinuotą pakopinės ETAS bei kombinuotą ETAS ir SKE–CO_2 ekstrakcijos schemas, pasiskirstė taip: BŠ nuo 0,15% (70°C, HX) iki 0,61% (140°C, HX), tuo tarpu BL nuo 5,2% (CO_2) iki 10,3 % (140°C, HX). Įvedus sistemos modifikatorių etanolį (ET), išeiga padidėjo iki 0,71% (BŠ) ir 8,15% (BL) (**3.1 lentelė**). Įdomu tai, jog keliant ETAS temperatūrą nuo 70 iki 140 °C lipofilinės frakcijos išeiga ženkliai padidėjo, nors BŠ atveju liko < 1 %

Acetonas (AC) buvo ypač efektyvus tirpiklis ekstrahuojant BŠ, – išeiga siekė 40 % nuo nuriebalintos žaliavos, tuo tarpu BL atveju, frakcijų išeigos siekė 4,71–11,4 %, skaičiuojant nuo augalo liekanos (AL) ir 4,47–10,2 % nuo pradinės augalo masės (PRAM). BL liekaną ektrahuojant poliniais tirpikliais – etanolio vandens mišiniu (ET/V) ir vandeniu (V), išeigos siekė 10,2–22,98 (AL) ir 7,95–17,1 (PRAM). Įdomu tai, jog ETAS temeperatūros pakėlimas iki 140 °C, neturėjo ženklios įtakos ($p < 0.05$) BŠ AC ekstrakto išeigai, nors kitu atveju, kai buvo ekstrahuojama acetonu SKE/CO_2 BŠ liekana, išeiga buvo ženkliai didesnė. BL atveju gauti priešingi rezultatai: keliant temperatūrą AC išeiga padvigubėjo.

Paskutinis ekstrakcijos žingsnis – ekstrakcija poliniais tirpikliais – ET/V bei V. ETAS BŠ atveju, keliant tempertūrą, ET/V išeiga padvigubėjo, kai tuo tarpu po SKE–CO_2/$ETAS_{70}$–AC–ET/V ekstrakcijos išeiga buvo mažesnė, lyginant su $ETAS_{70}$–HX/AC/ET/V. Panašūs rezultatai gauti ir ekstrahuojant ETAS bergenijos lapus (BL), tačiau SKE–CO2/ETAS–AC likučių ekstrakcija ET/V davė didesnę išeigą, lyginant su $ETAS_{70ir140}$–HX/AC/ET/V.

3.1. lentelė. *Bergenia crassifolia* L. ekstraktų, gautų panaudojant skirtingas ekstrakcijos schemas, išeigų (% w/w) ir antiokisdacinių savybių įvertinimas. ABTS ir DRSG reikšmės pateiktos μmol TE/g, BFJK – mg GRE/g ekstrakte (E) augalo liekanoje (AL) bei pradinėje augalo masėje (PRAM).

Ekstrakcijos procedūra	Tirpiklis	Išeiga		BFJK			ABTS			DRSG		
				E	AL	PRAM	E	AL	PRAM	E	AL	PRAM
Šaknys												
ETAS70°C	HX	0,15±0,12^{aA}	0,15	–	–		–	–		1315±119^{aA}	1,97^{aA}	1,97
	AC	41,2±3,0^{d}	41,1	187±4,80^{c}	76,8^{e}	76,9	2582±127^{d}	1064^{d}	1061	2332±208^{c}	961^{d}	958
	ET/V	19,2±1,1^{c}	11,3	167±4,91^{b}	32,0^{c}	18,9	1848±63,0^{c}	355^{b}	209	2583±182^{c}	496^{c}	292
	V	10,8±1,4^{b}	4,3	133±1,51^{a}	14,3^{a}	5,72	1388±98,1^{b}	150^{a}	150	1182±73,1^{a}	128^{a}	50,8
ETAS140°C	HX	0,61±0,0^{aC}	0,61	–	–		–	–		1267±16,4^{aA}	7,73,^{aC}	7,73
	AC	40,9±0,4^{d}	40,7	192±8,02^{c}	78,3^{e}	78,1	2422±63,1^{d}	991^{d}	986	2602±175^{c}	1064de	1059
	ET/V	39,6±1,5^{d}	23,2	153±6,44^{b}	60,6^{d}	35,5	1919±56,1^{c}	760^{c}	445	2258±203^{bc}	894^{d}	524
	V	10,2±1,9^{b}	1,93	125±5,81^{a}	12,7^{a}	2,41	1309±125^{b}	134^{a}	25,3	1281±111^{a}	131^{a}	24,7
SKE–CO_2	CO_2	0,37±0,1^{aB}	0,37	–	–		–	–		1356±37,1^{aA}	5,01^{aB}	5,01
	CO_2–ET	0,71±0,1^{aC}	0,71	–	–		–	–		2540±157^{cB}	18,0^{aD}	18,0
SKE–CO_2/ ETAS70°C	AC	46,6±1,3^{e}	46,4	195±10,50^{c}	90,9^{f}	90,5	2964±120^{e}	1381^{e}	1375	2539±250^{c}	1183^{e}	1178
	ET/V	15,6±1,5^{c}	8,27	161±3,71^{b}	25,1^{b}	13,3	1897±38,7^{c}	296^{b}	157	2100±75,1^{bc}	328^{b}	174
SKE–CO_2/ ETAS140°C	V	10,1±0,1^{b}	3,78	115±7,04^{a}	11,6^{a}	4,35	882±65,1^{a}	89,1^{a}	33,3	934±24,1^{a}	94,3^{a}	35,3

3.1. lentelė (tęsinys)

		Lapai										
ETAS70 °C	HX	5,72±0,50^{aA}	5,72	–	–		–	–		233±2,14^{aA}	13,3^{aA}	13,3
	AC	5,50±0,11^{a}	5,18	183±14,7^{c}	10,1^{a}	9,48	1987±154^{bc}	111^{a}	103	2193±179^{e}	121^{b}	114
	ET/V	10,8±0,34^{c}	9,59	207±10,1^{c}	22,4^{c}	19,9	2214±123^{bc}	239^{c}	212	2574±212^{f}	278^{cd}	247
	V	10,2±1,04^{c}	7,95	152±10,2^{bc}	15,5^{bc}	12,1	1720±17,6^{b}	175^{b}	137	1610±123^{d}	164^{b}	128
ETAS140°C	HX	10,3±0,31^{cC}	10,3	–	–		–	–		304±26,3^{aA}	31,3^{aC}	31,3
	AC	11,4±1,00^{c}	10,2	197±13,8^{c}	22,5	20,1	1582±61,1^{b}	180^{b}	161	2100±99,0^{e}	239^{c}	214
	ET/V	21,9±1,27^{e}	17,1	218±16,5^{cd}	47,7	37,3	2000±189^{bc}	438^{e}	342	1957±45,5^{e}	429^{e}	335
	V	19,4±0,77^{e}	10,9	128±7,81^{ab}	24,8	14,0	1596±40,1^{b}	309^{d}	174	1065±46,3^{b}	207^{c}	116
SKE–CO_2	CO_2	5,2±0,21^{aA}	5,2	–	–		–	–		399±30,7^{aAB}	20,7^{aB}	20,7
	CO_2–ET*	8,15±0,14^{bB}	8,15	–	–		–	–		1386±47,1^{cdC}	113^{cD}	113
SKE–CO_2/ ETAS70°C	AC	4,71±0,27^{a}	4,47	180±13,4^{bc}	8,48^{a}	8,05	1851±100^{b}	87,2^{a}	82,7	2001±141^{e}	94,2^{b}	89,4
	ET/V	15,4±0,11^{d}	13,9	223±11,7^{cd}	34,3^{d}	31,0	2302±94,4^{bc}	355^{d}	320	2188±64,1^{e}	337^{d}	304
SKE–CO_2/ ETAS140°C	V	22,9±0,58^{e}	17,1	104±8,91^{a}	23,8^{cd}	17,8	1116±58,8^{a}	256^{c}	191	1159±88,6^{bc}	265^{cd}	198

Reikšmės pateiktos, kaip trijų pakortojimų vidurkiai ± standartinis nuokrypis (n=3);
Skirtingos mažosios raidės, pateiktos stulpeliais (atskiri skaičiavimai šaknims ir lapams) nurodo reikšmingąskirtumą $p<0,05$; skirtingos Didžiosios raidės stulpeliuose (atskirai lapams bei šaknims) nurodo reikšmingą skirtumą tarp lipofilinių ekstraktų $p<0,05$.
HX–heksanas; AC–acetonas; ET/V–etanolis/vanduo (80/20); V–vanduo; CO_2–ET–anglies dioksidas su modifikatoriumi etanoliu.
*SKE–CO_2–ET ekstrakcija atlikta iš pradinės žaliavos ir gauta reikšmė neįtraukta į suminį PRAM skaičiavimą.

BŠ vandeninės (V) frakcijos išeiga siekė daugiau nei 10 % AL bei 1,93–3,79% visose taikytose ekstrakcijos schemose, kai tuo tarpu BL V frakcijos išeiga buvo apytikriai 2 kartus (skaičiuojat nuo AL) bei 1,37 karto (nuo PRAM) didesnė 140 °C nei 70 °C. Didžiausia V ekstrakto išeiga gauta taikant SKE–CO_2–$ETAS_{140}$ ekstrakciją – 22,9 ir 17,1 % atitinkamai, skaičiuojant nuo AL bei PRAM.

Taikant visas aukščiau paminėtas ekstrakcijos schemas, ekstraktų išeigos (suma visų gautų ekstraktų po pakopinės ekstrakcijos ir perskaičiuotos PRAM) buvo palyginti labai didelės, pavyzdžiui $ETAS_{140}$ suminė išeiga BŠ siekė net 66,44%, o BL – 48,5% PRAM.

3.1.2. Bendas fenolinių junginių bei antioksidacinių savybių įvertinimas storalapės bergenijos (*Bergenia crassifolia* L.) šaknų bei lapų ekstraktuose

Antiokisdacinis aktyvumas (AA) kito priklausomai nuo tirto augalo anatominių dalių, ekstrakcijos tirpiklio ir taikytos ekstrakcijos schemos. Pvz., bendras fenolinių junginių kiekis (BFJK) BŠ ir BL ekstraktuose svyravo nuo 115–195 iki 104–223 mg GRE/g (**3.1 lentelė**), ABTS ir BFJK BŠ ekstraktų reikšmės palaipsniui mažėjo, taikant didėjančio poliškumo tirpiklius – AC, ET/V bei V. Lipofilinės frakcijos, išgautos naudojant nepolinius tirpiklius (HX ir CO_2) netirtos, dėl jų mažo tirpumo metodo sistemoje. Visi tirti BŠ ekstraktai pasižymėjo didele radikalų sujungimo geba (RSG) DRSG (934–2602 µmol TE/g).

Didžiausios BŠ DRSG bei BL DRSG ir ABTS reikšmės gautos po $ETAS_{140}$ pakopinės ekstrakcijos.

3.1.3. Storalapės bergenijos (*Bergenia crassifolia* L.) antiokisdacinio aktyvumo įvertinimas kietoje frakcijoje taikant QUENCHER metodą

AA įvertinimas, tiek pradinėje žaliavoje tiek ekstraktuose, tiek augalo liekanoje po ekstrakcijos, suteikia išsamesnę informaciją apie augalo antioksidacinį patencialą bei apie įvairių išskyrimo procedūrų efektyvumą.

ABTS QUENCHER reikšmės BL buvo didesnės nei BŠ, išskyrus ETAS liekaną, gautą po ekstrakcijos ET/V ir V, kai tuo tarpu gautos BFJK ir DRSG reikšmės buvo labai skirtingos. Lyginant QUENCHER rezulatatus su rezultatais gautais tiriant ekstraktus, galime pastebėti, jog RSG šaknims bei lapams BFJK, ABTS ir DRSG metoduose, tiriant ekstraktus ir rezultatus perskaičiavus 1 g PRAM buvo labai logiški lyginant su pradinės žaliavos QUENCHER rezultatais. Pavyzdžiui, BFJK QUENCHER BŠ pradinės žaliavos reikšmė buvo 224 mg GRE/g PRAM, o suma BFJK, gauta po pakopinės ekstrakcijos $ETAS_{140}$ ir perskaičiuota 1g PRAM buvo tik 13 % mažesnė – 194,2 mg GRE/g PRAM (Σ_{TPC} = 78,1 + 35,5 + 2,41 + 78,2). Tikėtina, jog:

(1) taikant pakopinę ekstrakciją didžioji dauguma junginių buvo išekstrahuoti pirmuosiuose ekstrakcijos žingsniuose;

(2) dalis aktyviųjų junginių liko sujungti augalo matricoje su kitais junginiais.

Tradiciniai RSG metodai taikomi skystąjai frakcijai, bei QUENCHER procedūra – kietąjai frakcijoms, leidžia pilniau apibūdinti augalo masės antioksidacinį potencialą bei įvairių išskyrimo metodų efektyvumą.

3.1.4. Kiekybinis bioaktyvių junginių įvertinimas storalapės bergenijos (*Bergenia crassifolia* L.) ekstraktuose, naudojant efektyviąją skysčių chromatografija–masių spektroskopiją

Fitocheminės ekstraktų sudėties įvertinimas atliktas, taikant efektyviąją skysčių chromatografiją–masių spektroskopiją. Junginiai idetifikuoti nustatant tikslią jų masę bei išėjimo laiką. Pagrindiniai bioaktyvūs junginiai tiek BŠ, tiek BL ekstraktuose buvo bergeninas, katechino galatas, elago rūgštis bei kvercetino–3–β–D–gliukozidas. Dalies chromatogramoje užfiksuotų pikų nepavyko identifikuoti, kadangi masės spektrų bibliotekoje buvo pateikta daug junginių, atitinkančių tą pačią masę.

Pagrindinis kiekybinis junginys tiek BŠ tiek BL ekstraktuose buvo bergeninas (**3.2 lentelė**). Bergenino kiekis, išgautas iš BŠ, svyravo 45,24–50,35 mg/g PRAM ribose ir jis buvo ženkliai didesnis nei BL – 3,69–4,51 mg/g PRAM. Palyginti, didelis kiekis bergenino identifikuotas SKE–CO_2 ekstrakte, tačiau šio ekstrakto išeiga buvo labai maža (0,71%), todėl ir bergenino kiekis BL PRAM tesiekė 0,92 mg/g. Tačiau įvedus sistemoje modifikatorių etanolį (ET), ekstrakto išeiga ženkliai padidėjo (8,15%) ir beregnino kiekis ekstrakte siekė 89 mg/g, todėl šis ekstrakcijos metodas galėtų būti panaudojamas bergenino išskyrimui iš BL pramoniniais tikslais. Taip pat palyginti dideli kiekiai nustatyti ir kvercetino–3–β–D–gliukozido bei katechino galato tiek BŠ tiek BL, o galo rūgštis aptikta tik BL. AC dėl didelės išeigos, buvo pats efektyviausias tirpiklis bergenino ir kitų junginių išskirtų iš BŠ. Pavyzdžiui, ekstrakcija AC atitinkamai išskyrė 62,0–86,6%, 75,5–98,5% ir 72,8–94,0% viso bergenino kiekio, kvercetino–3–β–D–gliukosido ir katechino galato kiekio po pakopinės ekstrakcijos.

Taip pat paminėtina tai, jog junginių išskyrimo efektyvumas buvo didesnis taikant $ETAS_{140}$: bergenino, kvercetino–3–β–D–gliukosido ir katechino galato kiekiai atitinkamai buvo 11,3, 39,6 and 16,5% didesni lyginant su $ETAS_{70}$.

Apibendrinant, galima teigti, jog šie tyrimai rodo, kad BŠ esantys junginiai yra stipriai įkapsuliuoti augalo matricoje ir jie išlieka stabilūs netgi auštose ekstrakcijos temepratūrose. Tai labai svarbu norint pasirinkti tinkamą ekstrakcijos procedūrą, Temepratūrinis režimas bergenino ir kvercetino–3–β–D–gliukosido ekstrakcijai iš BL nebuvo toks svarbus, o elago rūšties atveju minėtų junginių kiekiai 140 °C buvo netgi gerokai mažesni lyginant su 70 °C. Tikėtina, jog elago rūgštis yra nestabili aukštuose temperatūriniuose režimuose, todėl BL ekstrakcija turėtų būti atliekama, taikant žemesnę temperatūrą.

Įvairūs fenoliniai junginiai bei flavonoidai, pavyzdžiui, bergeninas plačiai naudojamas Ajurvedinėje medicinoje. Jis pasižymi antivirusiniu, priešgrybeliniu, priešuždegiminiu, priešvėžiniu, antidiabetiniu ir kitais teigiamais poveikiais.

3.2. lentelė. Pagrindinių *Bergenia crassifolia* L. fenolinių junginių kiekiai ekstraktuose (E) augalo liekanoje (AL) ir pradinėje augalo masėje (PRAM), mg/g

Bandinys	Tirpiklis	Bergeninas			(–)–Katechino galatas			Elago rūgštis			Kvercetino–3–*β*–D–gliucosidas		
		E	PRAM	**ΣPRAM**	E	PRAM	**ΣPRAM**	E	PRAM	**ΣPRAM**	E	PRAM	**ΣPRAM**
							Šaknys						
ETAS 70°C	HX	75,7±2,0	0,11		0,12±0,00			–	–	–	–		
	AC	81,3±4,2	33,4	**45,24**	16,2±0,10	6,66	**8,06**	–	–	–	21,3±0,1	8,75	**10,63**
	ET/V	74,9±3,8	8,46		12,4±0,24	1,40		–	–	–	16,6±0,3	1,88	
	V	76,1±2,4	3,27		–			–	–	–	–		
ETAS 140°C	HX	63,0±2,4	0,38		–			–	–	–	–		
	AC	76,6±3,1	31,2	**50,35**	16,8±0,91	6,84	**9,39**	–	–	–	27,4±1,0	11,2	**14,84**
	ET/V	75,5±4,5	17,5		11,0±0,12	2,55		–	–	–	15,7±0,6	3,64	
	V	65,8±3,1	1,27		–			–	–	–	–		
SKE–CO_2	CO_2–ET*	130±7,1	0,92		0,03±0,00	<0,01		–	–	–	–		
	CO_2	56,5±2,5	0,21	**48,73**	–		**8,0**	–	–	–	–		**10,17**
SKE–CO_2/ ETAS70°C	AC	92,6±5,6	43,0		16,2±0,01	7,52		–	–	–	21,5±0,9	10,02	
	ET/V	46,0±1,8	3,80		5,81±0,05	0,48		–	–	–	1,84±0,0	0,15	
SKE–CO_2/ ETAS140°C	V	45,4±2,2	1,72		–			–	–	–	–		

3.2. lentelė (tęsinys)

		Lapai											
ETAS 70 °C	HX	–	–			–			–			–	–	
	AC	21,2±1,1	1,10	**3,69**	0,17±0,00	0,01	**0,02**	0,04±0,00	<0,01	**0,02**	35,1±1,0	1,82	**4,46**
	ET/V	16,5±0,1	1,58		0,12±0,00	0,01		0,04±0,00	<0,01		27,5±1,0	2,64	
	V	12,7±0,4	1,01			–		2,56±0,01	0,20			–	–	
ETAS 140°C	HX		–			–			–			–	–	
	AC	16,7±0,1	1,70	**4,0**	0,16±0,00	0,02	**0,06**	0,06±0,00	0,01	**0,02**	24,2±1,0	2,47	**3,94**
	ET/V	10,2±0,5	1,04		0,25±0,01	0,04		0,07±0,00	0,01		8,57±0,0	1,47	
	V	7,35±0,0	1,26		–	–		–	–			–		
SKE–CO_2	CO_2–ET*	89,0±4,2	7,25		–			–			–	–	
	CO_2	–			–			–			–	–	
SKE–CO_2/ ETAS70°C	AC	28,5±1,1	1,27	**4,51**	0,33±0,01	0,015	**0,022**	0,05±0,00		**0,01**	43,5±1,2	1,94	**3,18**
	ET/V	12,3±0,1	1,71		0,05±0,00	0,007		0,07±0,00	0,01		8,89±0,0	1,24	
SKE–CO_2/ ETAS140°C	V	8,96±0,1	1,53		–			–			–	–	

Reikšmės pateiktos, kaip trijų pakartojimų vidurkiai ± standartinis nuokrypis (n=3);
HX–heksanas; AC–acetonas; ET/V–etanolis/vanduo (80/20); V–vanduo; CO_2–ET–anglies dioksidas su modifikatoriumi etanoliu.
*SKE–CO_2–ET ekstrakcija atlikta iš pradinės žaliavos ir gauta reikšmė neįtraukta į suminį PRAM skaičiavimą.

3.1.5. Storalapės bergenijos (*Bergenia crassifolia* L.) šaknų bei lapų ekstraktų antioksidacinis efektyvumas, taikant oksipres ir rancimat metodus

Visi tirti ekstraktai pasižymėjo rapsų aliejaus (RA) bei emulsijų (EM) stabilizavimo efektu – stabilumo koeficientas (SK) siekė 1,02–1,62. Didžiausias antioksidacinis stabilumas nustatytas BŠ ir BL su AC ir ET/V ekstraktų priedais: SK 1,38–1,58 (AC) ir 1,43–1,62 (ET/V).

3.2. Grikių (*Fagopyrum Esculentum Moench.*) žiedynų biorafinavimas, taikant aukšto slėgio ekstrakcijos metodus, antiokisdacinių savybių bei pagrindinių aktyviųjų junginių įvertinimas

3.2.1. Grikių (*Fagopyrum Esculentum Moench.*) žiedynų išeigų įvertinimas

Grikių žiedų frakcijų išeigos kito priklausomai nuo ekstrakcijai naudoto tirpiklio poliškumo bei ekstrakcijos temperatūrinio režimo. Jei CO_2 ir HX išeigos siekė 1,78 – 3,15 % PRAM (**3.3 lentelė**), tai 10 proc. etanolio priedas SKE–CO_2 ekstrakcijoje išeigą padidino 48%. Didėjant tirpilio poliškumui (AC, ET/V and V) didėjo ir išeigos, tačiau jos taip pat labai priklausė nuo proceso temperatūros: AC, ET/V ir V ekstraktų išeigos $ETAS_{140}$ buvo 60, 50,3 ir 220,3 % didesnės lyginant su $ETAS_{70}$. Suminės išeigos $ETAS_{70}$ ir $ETAS_{140}$ buvo 37,02 ir 64,05%. Suminė ekstraktų išeiga, įtraukiant į procesą ir SKE–CO_2 buvo 51,38 %.

3.2.2. Bendas fenolinių junginių bei antioksidacinių savybių įvertinimas grikių (*Fagopyrum Esculentum Moench.*) žiedynų ekstraktuose

AA reikšmių pasiskirstymas (**3.3 lentelė**) priklausė nuo taikyto metodo bei ekstrakcijos procedūros. AA lyginant $ETAS_{140}$ su $ETAS_{70}$ ir ekstrahuojant tuo pačiu tirpikliu buvo didesnis nuo 1,1 (BFJK–ETAS–ET/V) iki 3 kartų (DRSG–ETAS–V), tačiau įvertinus tai, jog didžiausios suminės išeigos gautos $ETAS_{140}$, tai BFJK skaitinė reikšmė išreikšta PRAM buvo didesnė nei $ETAS_{70}$ (145,5 vs 101,6 GRE/g PRAM). Suminė SKE–CO_2–ETAS ekstraktų BFJK išraiška siekė 124,1 GRE/g PRAM. Panaši tendencija pastebėta ir DRSG ir ABTS metoduose, pavyzdžiui, $ETAS_{140}$ ekstraktai pasižymėjo silpnesniu AA lyginant su $ETAS_{70}$ ABTS metode, nors suminė $ETAS_{140}$ ABTS reikšmė buvo 1,46 karto didesnė nei $ETAS_{70}$. Tik DRSG HX buvo 1,4 karto didesnė $ETAS_{140}$ temperatūriniame režime. Įdomu ir tai, jog SFE–CO_2–ETAS DRSG reikšmės perskaičiuotos PRAM buvo didesnės už $ETAS_{70}$ ir $ETAS_{140}$. Polinio tirpiklio etanolio taikymas SKE–CO_2 padidino ekstrakto DRSG 25 %. Lyginant V ekstraktus su AC ir ET/V ekstraktais, pastarieji buvo silpnesni antioksidantai, su keliomis išimtimis, priklausomai nuo ekstrakcijos temepratūros ir taikyto tyrimo metodo.

3.2.3. Antiokisdacinio aktyvumo įvertinimas grikių (*Fagopyrum esculentum* Moench) žiedynų kietoje frakcijoje taikant QUENCHER metodą

Pakopinė ekstrakcija taikant didėjančio poliškumo tirpiklius, palaipsniui sumažino liekanų AA, nors galutinė liekana dar pasižymėjo antioksidaciniu

3.3. lentelė. *Fagopyrum esculentum* Moench. žiedynų ekstraktų, gautų panaudojant skirtingas ekstrakcijos schemas, išeigų % ir antiokisdacinių savybių įvertinimas. ABTS ir DRSG reikšmės pateiktos µmol TE/g, BFJK – mg GRE/g ekstrakte (E) augalo liekanoje (AL) bei pradinėje augalo masėje (PRAM)

Bandinys	Tirpiklis	Išeiga		BFJK			ABTS			DRSG		
		AL	PRAM	E	AL	PRAM	E	AL	PRAM	E	AL	PRAM
$ETAS_{70}$	HX	3,10±0,12^{aC}	3,10^{ab}							714±25^{a}	22.1^{ab}	22,1^{ab}
	AC	12,2±0,91^{c}	11,8^{b}	309±17,2^{c}	37,7^{b}	36,5^{c}	2401±100^{d}	293^{c}	283^{c}	2087±110^{e}	255^{e}	246^{d}
	ET/V	21,7±1,20^{d}	18,5^{c}	292±19,3^{c}	63,4^{d}	54,0^{d}	2211±142^{d}	480^{e}	409de	2114±100^{c}	459^{g}	391de
	V	6,82±0,74^{b}	4,54^{ab}	245±17,0^{b}	16,7^{a}	11,1^{a}	1779±95^{b}	121^{a}	80,8^{a}	1974±125^{c}	135^{c}	89.6^{c}
	Σ		**37,02**			**101,6**			**772,8**			**748,7**
$ETAS_{140}$	HX	3,15±0,22^{aC}	3,15^{ab}	–	–	–	–	–	–	979±47^{ab}	30,8^{b}	30,8^{b}
	AC	19,5±1,01^{d}	18,9^{c}	228±10,1^{b}	44,5^{bc}	43,1^{cd}	1797±122^{b}	350^{d}	340^{d}	1514±99^{b}	295^{ef}	286^{d}
	ET/V	35,6±2,71^{f}	27,8^{e}	272±15,0^{bc}	96,8^{e}	75,6^{f}	2124±154^{d}	756^{g}	590^{e}	1002±29^{ab}	357^{f}	279^{d}
	V	28,3±1,88^{e}	14,2^{b}	189±11,7^{ab}	53,5^{c}	26,8^{b}	1383±84^{a}	391^{d}	196^{b}	651±30^{a}	184^{d}	92.4^{c}
	Σ		**64,05**			**145,5**			**1126**			**688,2**
SKE	CO_2	1,78±0.17^{aA}	1,78^{a}	–	–	–	–	–	–	807±37^{ab}	14,4^{a}	14,4^{a}
SKE	CO_2/ET*	2,63±0.11^{aB}	2,63^{ab}	–	–	–	–	–	–	1012±56^{ab}	26,6^{ab}	26,6^{ab}
SKE–	AC	12,6±1.04^{c}	12,4^{b}	312±12,4^{c}	39,3^{b}	38,7^{c}	2197±59^{d}	214^{b}	272^{c}	2071±100^{c}	261^{e}	257^{d}
CO_2/$ETAS_{70}$	ET/V	26,5±1.54^{de}	22,7^{d}	265±11,8^{bc}	70,2^{d}	60,2^{de}	2371±102^{d}	628^{f}	538^{e}	2000±158^{c}	530^{h}	454^{e}
SKE–	V	22,9±0.50^{d}	14,5^{b}	174±14,6^{a}	39,8^{b}	25,2^{b}	1237±105^{a}	283^{c}	179^{b}	672±32^{a}	154^{c}	97,4^{c}
CO_2/$ETAS_{140}$												
	Σ		**51,38**			**124,1**			**989**			**822,8**

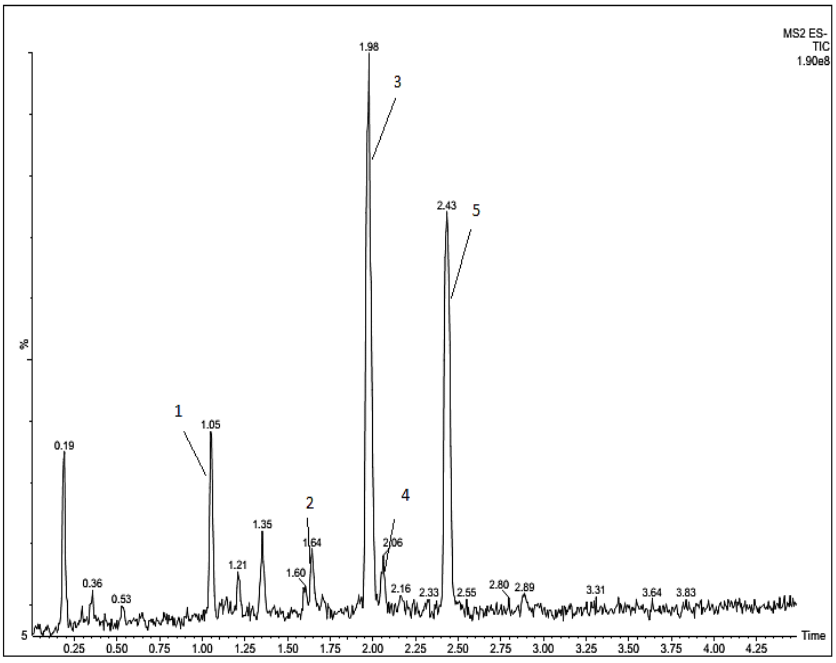
Reikšmės pateiktos, kaip trijų pakortojimų vidurkiai ± standartinis nuokrypis (n=3);
Skirtingos mažosios raidės, pateiktos stulpeliais nurodo reikšmingą skirtumą $p<0,05$;
Skirtingos didžiosios raidės stulpeliuose nurodo reikšmingą skirtumą tarp lipofilinių ekstraktų $p<0,05$.
HX–heksanas; AC–acetonas; ET/V–etanolis/vanduo (80/20); V–vanduo; CO_2–ET–anglies dioksidas su modifikatoriumi etanoliu.
*SKE–CO_2–ET ekstrakcija atlikta iš pradinės žaliavos ir gauta reikšmė neįtraukta į suminį PRAM skaičiavimą.

aktyvumu. BFJK, ABTS ir DRSG AA reikšmės po paskutinio $ETAS_{70}$ ekstrakcijos žingsnio buvo 3,2; 2,2; 1,8 karto mažesnis lyginant su pradinės žaliavos AA.

Galima teigti, jog $ETAS_{140}$ ekstrakcijos schema efektyviausiai išekstrahavo AA pasižyminčius junginius – $ETAS_{140}$–V liekanų AA reikšmės buvo mažiausios ir sumažėjo nuo pradinės žaliavos atitinkamai 10,6 (BFJK); 6,5 (ABTS) ir 5,0 (DRSG) karto. Koreliacija tarp BFJK ir RSG QUENCHER metode buvo netgi didensė lyginant su ekstraktų: BFJK vs ABTS 0,92 ir BFJK vs. DRSG R^2=0,94.

3.2.4. Kiekybinis bioaktyvių junginių įvertinimas grikių (*Fagopyrum esculentum* Moench.) žiedynų ekstraktuose, naudojant efektyviąją skysčių chromatografija–masių spektroskopiją

Turimų literatūros šaltinių duomenimis citrinų rūgštis, kvercetino arabinozidas, galaktozidas, izoorientinas ir miricitrinas grikių žiedynuose nebuvo aptikti iki šiol.



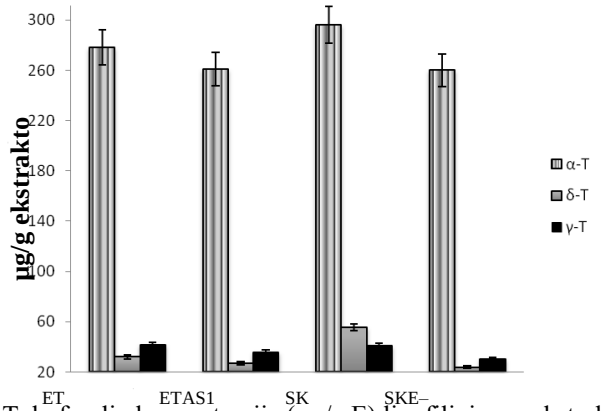
3.1 Pav. Pagrindiniai fenoliniai *Fagopyrum esculentum* Moench.junginiai $ETAS_{70}$ ET/V. 1 – chlorogeno r., 2 – izoorientinas, 3 – rutinas, 4 – kvercetino galaktozidas, 5 – kvercitrinas

Rutinas – pagrindinis kiekybinis junginys aptiktas grikių žiedynų nelipofiliniuose ekstraktuose (35,38–56,8 mg/g PRAM) (**3.1 pav.**). Po rutino seka kvercitrinas, kurio kiekis ekstrakte siekė daugiau nei 88 mg/g E ($SKE–CO_2$–$ETAS_{70}$–AC). Kvercitrinas sudarė 1,83–4,2 % PRAM nuo visų išskirtų junginių, taikant pakopinę ekstrakciją. Ypač didelė rutino koncentracija nustatyta ET/V ir AC ekstraktuose, išskyrus ETAS 140–ET/V; nors ET/V dėl itin didelės išeigos buvo pats efektyviausias tirpiklis daugumos junginių išskyrimui, skaičiuojant PRAM. Pavyzdžiui, ETAS140–ET/V sudarė 45,0 %, 45,4 %, 47,4 %, 43,5 %, ir 44,2 %, atitinkamai viso chlorogeno, citrinų rūgšties, kvercetino arabinozido, kvercitrino ir

rutino kiekio 1g PRAM. Ekstrakcijos temperatūra taip pat turėjo įtakos junginių išskyrimo efektyvumui iš grikių žiedynų. ETAS 140 °C temperatūrinis režimas buvo efektyvesnis už ETAS 70 °C 40,5 %, 10,6 %, 46,3 % ir 60,5 % atitinkamai vertinant chlorogeno, citrinos rūgštį, kvercitriną ir rutiną. Suminis išskirtų junginių kiekis taikant ETAS 70, ETAS 140 ir kombinuotą SKE–CO_2–ETAS schemas sudarė atitinkamai 80,69; 116,16 ir 83,76 mg/g PRAM. Tai rodo, jog trumpas ekstrakcijos laikas aukštoje tempertaūroje yra efektyviausias ektrakcijos procesas, kuris leidžia išekstrahuoti didelius kiekius fitojunginių nepakeičiant jų cheminių struktūrų. Miricitrinas ir izoorientinas aptikti mažiausiais kiekiais tirtuose grikių žiedynuose – atitinkamai 0,08–0,11 mg/g E ir 0,06–0,91 mg/g E.

3.2.5. Tokoferolių kiekio įvertinimas grikių (*Fagopyrum esculentum* Moench.) žiedynų lipofiliniuose ekstraktuose

Gauta tiesinė priklausomybė tarp tyrimams naudotų standartinių įvairios koncentracijos tokoferolių bandinių ir piko ploto, buvo labai aukšta: α–T, R^2 = 0,999; γ–T, R^2 = 0,999; δ–T, R^2 = 0,999. Suminė tokoferolių koncentracija lipofiliniuose ekstraktuose siekė 1381 µg/g E (**3.2 pav.**). Didžiausias kiekis α–T aptiktas SKE–CO_2 ekstrakte. Jo kiekis svyravo nuo 260 iki 296 µg/g ekstrakte. Tai patvirtino anksčiau publikuotų duomenų rezultatus.



3.2. Pav. Tokoferolių koncentracija (µg/g E) lipofiliniuose ekstraktuose; HX–heksanas; CO_2–ET – anglies dioksidas su modifikatoriumi etanoliu (10%). Reikšmės pateiktos, kiap trijų pakartojimų vidurkiai (n=3).

Modifikatoriaus etanolio įvedimas į ekstrakcijos procesą sumažino tokoferolių koncentraciją apie 1,3–1,5 karto, tai galima paiškinti tuo, jog ekstrakte esantys tokoferoliai prasiskiedė su mažiau lipofiliniais junginiais (ET padidino ekstrakto išeigą 1,48 karto).

3.2.6. Grikių (*Fagopyrum esculentum*) žiedynų ekstraktų antioksidacinis efektyvumas, taikant oksipres ir rancimat metodus.

Dėl lipofilinių (tokoferoliai) ir aukšto poliškumos (hidrofilinių) antiokisdantų (flavanoidai, fenolinės rūgštys) grikių žiedynų ekstraktai tirti tiek gryname rapsų aliejuje (RA) tiek ir emulsijose (EM) riebalai/vanduo (70/30), pridedant 0,5 % ekstrakto priedą.

3.4. lenetelė. *Fagopyrum esculentum* Moench.žiedynų ekstraktų priedų (0,5%) antiokisdacinis efektyvumas rapsų aliejuje (RA) ir emulsijose (EM) 120 °C temeperatūroje.

	Oksipres 120 °C				Rancimat 120 °C	
Ekstraktų priedai	RA		EM		EM	
	IP	SK	IP	SK	IP	SK
RA (Kontrolė)	2,18±0,01	1,00	–	–	–	–
E (Kontrolė)	–		2,64	1,00	9,24	1
$ETAS_{70}$ HX	2,62±0,01	1,20	–	–	–	–
SKE–CO_2	2,74±0,02	1,26	–	–	–	–
$ETAS_{70}$AC	–	–	3,95±0,02	1,50	11,3±0,04	1,23
$ETAS_{70}$–ET/V	–	–	6,15±0,09	2,34	18,0±0,03	1,95
$ETAS_{140}$–VW	–	–	3,89±0,01	1,47	10,9±0,01	1,18

AA pateiktas, apskaičiavus indukcinį period (IP) bei stabilumo koeficientą (SK)

Visi GŽ ekstraktai pasižymėjo RA ir EM stabilizavimo efektu: SK kito nuo 1,20 iki 2,34. Didžiausiu AA aliejuje pasižymėjo ET/V ekstraktai (SK 2,34); šie rezultatai sutapo ir su dideliu antiokisdaciniu aktyvumu, tirtu *in vitro*.

3.3. Europinės rykštenės (*Solidago virgaurea* L.) biorafinavimas, taikant aukšto slėgio ekstrakcijos metodus, antiokisdacinių savybių bei pagrindinių aktyviųjų junginių įvertinimas

3.3.1 Europinės rykštenės (*Solidago virgaurea* L.) išeigų įvertinimas

Lipofilinių rykštenės lapų (RL) ekstraktų išeigos svyravo nuo 2,40 % PRAM (SKE–CO_2) iki 5,89 % PRAM (PLE_{140}–HX). Modifikatoriaus etanolio panaudojimas SKE–CO_2 ekstrakcijos schemoje, padidino ekstrakto išeigą apytikriai 2,3 karto (5,52 %) (**3.5 lentelė**).

Didėjančio poliškumo tirpikliais AC, ET/V ir V gautos išeigos buvo ženkliai didesnės lyginant su nepoliniais tirpikliais ir labai priklausė nuo ekstrakcijos temperatūrinio režimo. Išeigos, gautos 140 ° C temparatūroje, tiek AC, tiek ET/V bei V buvo atitinkamai 48, 67 and 17 % didesnės nei 70 ° C temperatūroje ($ETAS_{70}$). Suminė ekstraktų išeiga $ETAS_{140}$ buvo didžiausia (63,79% PRAM) ir net 49 % didesnė lyginant su $ETAS_{70}$. Taikant kombinuotą ekstrakcijos schemą SKE–CO_2–ETAS gauta suminė ektraktų išeiga sudarė 55,10 % PRAM.

3.3.2. Bendas fenolinių junginių bei antioksidacinių savybių įvertinimas Europinės rykštenės (*Solidago virgaurea* L.) lapų ekstraktuose

Iš pateiktų antiokisdacinių RL ekstraktų rezultatų (**3.5 lentelė**) matyti jog, temperatūrinis režimas ($ETAS_{70}$ vs. $ETAS_{140}$) turėjo didelę įtaką antiokisdaciniam ekstraktų aktyvumui – radikalų surišimo geba ABTS, DRSG ir BFJK sumažėjo nuo 1,02 (BFJK–ETAS–ET/V) iki 2,20 (DRSG–ETAS–V) karto, tačiau dėl ženkliai didesnių išeigų $ETAS_{140}$ temperatūriniame režime, suminės BFJK ir ABTS reikšmės PRAM buvo atitinkamai 37 % ir 31 % didesnės lyginant su $ETAS_{70}$. BFJK reikšmės svyravo nuo 98 (SKE–CO_2/$ETAS_{140}$V) iki 185 ($ETAS_{70}$ AC) mgGRE/g ekstrakto

($p<0.05$). ABTS ir DRSG didžiausias RSG pasižymėjo $ETAS_{70}$–AC–ET/V; $ETAS_{140}$–AC, ET/V bei SKE–CO_2/ETAS–AC ET/V ekstraktai ($p<0,05$).

3.3.3. Europinės rykštenės (*Solidago virgaurea* L.) antiokisdacinio aktyvumo įvertinimas kietoje frakcijoje taikant QUENCHER metodą

Kietosios frakcijos antioksidacinis aktyvumas laipsniškai mažėjo, taikant pakopinę ekstrakciją. Lyginant BFJK, ABTS ir DRSG reikšmes su pradinės žaliavos vertėmis reikšmingo skirtumo nepastebėta ($p>0,05$), greičiausiai dėl mažų ekstrakcijos išeigų. Reikšminiai skirtumai nustatyti tarp pradinės žaliavos ir paskutinės ekstrakcijos pakopos liekanos. Pavyzdžiui, pradinės žaliavos (PŽ) DRSG AA lyginant su $ETAS_{70}$–V, $ETAS_{140}$–V bei SKE–ETAS–V skyrėsi atitinkamai 2,37; 1,92 ir 2,39 karto. Stipri koreliacija nustatyta tarp BFJK ir ABTS AA ($R^2=0,87$) bei BFJK ir DRSG ($R^2=0,91$). Nors didelė dalis AA pasižyminčių junginių buvo išekstrahuota taikant pakopinę ekstrakciją skirtingo poliškumo tirpikliais, tačiau dalis junginių vis dar liko sujungti kietojoje fazėje. Taikant tradicinę bei QENCHER procedūras leidžia išsamiau apžvelgti AA tiek augalo ekstraktuose tiek liekanoje.

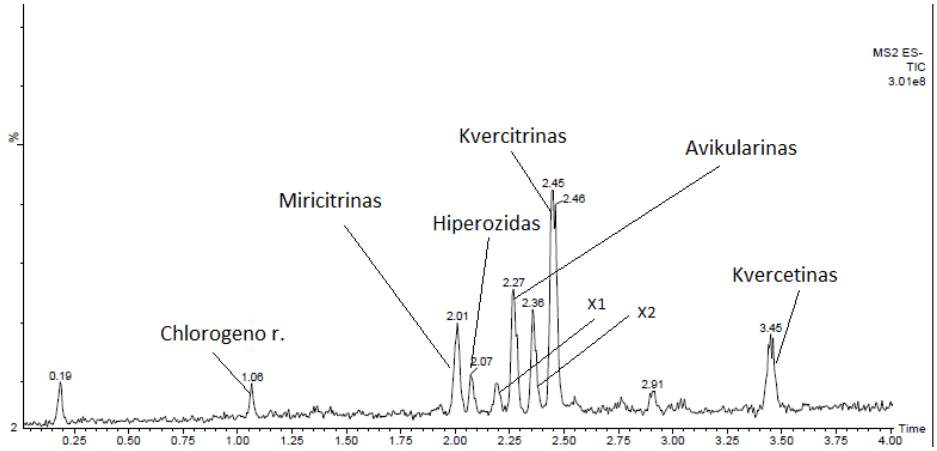
3.5. lentelė. *Solidago virgaurea* L. lapų ekstraktų, gautų panaudojant skirtingas ekstrakcijos schemas, išeigų % (w/w) ir antiokisdacinių savybių įvertinimas. ABTS ir DRSG reikšmės pateiktos µmol TE/g, BFJK – mg GRE/g ekstrakte (E) bei pradinėje augalo masėje (PRAM).

Bandinys	Tirpiklis	Išeiga	BFJK		ABTS,		DRSG	
		PRAM	E	PRAM	E	PRAM	E	PRAM
PLE_{70}	HX	$3,86^{aB}$					$677±13,1^{a}$	$26,1^{a}$
	AC	$11,7^{c}$	$185±10,7^{c}$	$21,6^{b}$	$1293±101^{b}$	151^{b}	$2497±111^{e}$	292^{c}
	ET/W	$17,3^{e}$	$177±9,81^{c}$	$30,6^{c}$	$1338±98,1^{b}$	231^{c}	$2814±110^{f}$	487^{e}
	W	$10,0^{c}$	$142±5,70^{b}$	$14,2^{a}$	$1098±57,7^{ab}$	110^{a}	$1985±95,7^{d}$	199^{b}
	Σ	**42,87**		**66,4**		**492**		**1004,1**
PLE_{140}	HX	$5,89^{bC}$	–	–	–	–	$540±28,7^{a}$	$31,8^{a}$
	AC	$17,3^{e}$	$163±9,10^{bc}$	$28,2^{c}$	$1200±67,9^{b}$	208^{c}	$1939±91,4^{d}$	335^{c}
	ET/W	$28,9^{g}$	$174±8,71^{c}$	$50,3^{e}$	$1153±94,5^{b}$	333^{e}	$1579±89,1^{c}$	456^{e}
	W	$11,7^{c}$	$106±7,40^{a}$	$12,4^{a}$	$870±41,7^{a}$	102^{a}	$904±61,8^{b}$	106^{ab}
	Σ	**63,79**		**90,9**		**643**		**928,8**
SFE	CO_2	$2,40^{aA}$	–	–	–	–	$511±25,4^{a}$	$12,3^{a}$
SFE	CO_2/ET*	$5,52^{abC}$	–	–	–	–	$804±39,7^{ab}$	$44,4^{ab}$
SFE–CO_2/PLE_{70}	AC	$12,9^{cd}$	$169±10,1^{bc}$	$21,8^{b}$	$1172±84,5^{b}$	151^{b}	$2807±100^{f}$	362^{c}
	ET/W	$24,1^{f}$	$142±9,00^{b}$	$34,2^{cd}$	$1169±44,7^{b}$	282^{d}	$2583±110^{ef}$	623^{f}
SFE–CO_2/PLE_{140}	W	$15,7^{e}$	$98.0±5,47^{a}$	$15,4^{a}$	$950±31,3^{ab}$	149^{b}	$1815±92,2^{cd}$	285^{c}
	Σ	**55,10**		**71,4**		**582**		**1282,3**

Reikšmės pateiktos, kaip trijų pakortojimų vidurkiai ± standartinis nuokrypis (n=3);
Skirtingos mažosios raidės, pateiktos stulpeliais) nurodo reikšmingą skirtumą $p<0,05$;
Skirtingos didžiosios raidės stulpeliuose nurodo reikšmingą skirtumą tarp lipofilinių ekstraktų $p<0,05$;
HX–heksanas; AC–acetonas; ET/V–etanolis/vanduo (80/20); V–vanduo; CO_2–ET–anglies dioksidas su modifikatoriumi etanoliu;
*SKE–CO_2–ET ekstrakcija atlikta iš pradinės žaliavos, o gauta reikšmė neįtraukta į suminį PRAM skaičiavimą.

3.3.4. Kiekybinis fitojunginių įvertinimas *Solidago virgaurea* L. Lapų ekstraktuose naudojant efektyviąją skysčių chromatografija–masių spektroskopiją

Nustatyta, jog aptiktas kvercitrino kiekis visuose rykštenės lapų ekstraktuose buvo didžiausias iš visų identifikuotų junginių (išskyrus lipofilinius ekstraktus) (**3.3 pav.**).



3.3. pav. Pagrindiniai bioaktyvūs junginiai aptikti *Solidago virgaurea* L. lapų ekstraktuose ($ETAS_{70}$–AC)

Didelis jo kiekis nustatytas $ETAS_{140}$–ET/V (76,0 mg/g E), $ETAS_{140}$–AC (80,9 mg/g E) and $ETAS_{70}$–AC (84,6 mg/g E). $ETAS_{140}$ET/V dėl itin didelės išeigos buvo pats efektyviausias tirpiklis kvercitrino ir kitų pagrindinių junginių ekstrakcijoje iš RL, išskyrus kvercetiną, kur $ETAS_{140}$–AC išekstrahavo 20,9% daugiau lyginant su $ETAS_{140}$–ET/V. Pavyzdžiui, ekstrakcija $ETAS_{140}$–ET/V išekstrahavo atitinkamai 69%, 41%, 52%, 52 %, 39%, 50 and 54% viso nustatyto chlorogeno, citrinos rūgšties kvercetino arabinopiranozido, hiperozido, kvercetino ir miricitrino kiekio.Taip pat nustatyta, jog identifikuotų junginių išskyrimo efektyvumas iš RL buvo gerokai didesnis, taikant aukštesnį temperatūrinį režimą. Taikant $ETAS_{140}$ išskirtų junginių kiekis buvo nuo 1,5 (chlorogeno, citrinų rūgštis) iki 8,9 (rutinas) karto didesnis lyginant su $ETAS_{70}$.

Antrasis kiekybiškai svarbus junginys – hiperosidas (kvercetino–3–D–galaktosidas), jo suminis kiekis svyravo nuo 17,2 ($ETAS_{70}$) iki 28,11 mg/g PRAM ($ETAS_{140}$). Taip pat ženklus kiekis avikularino (kvercetino 3–O–α–L–arabinopiranozido) nustatytas po pakopinės ekstrakcijos. Jis svyravo nuo 9,64 ($ETAS_{70}$) iki 15,49 mg/g PRAM ($ETAS_{140}$).

Suminis chlorogeno rūgšties kiekis, kito priklausomai nuo temperatūros ir svyravo tarp 10,24 ir 15,79 mg/g PRAM. Citrinos rūgštis pagal turimus literatūros duomenis, nustatyta RL lapų ekstraktuose pirmą kartą, jos kiekis kito tarp 7,86 ir 19,72 mg/g PRAM.

3.3.5. Tokoferolių kiekio įvertinimas Europinės rykštenės (*Solidago virgaurea* L.) lapų lipofiliniuose ekstraktuose

Didžiausias suminis tokoferolių kiekis nustatytas $ETAS_{70}$–HX ekstrakte (202,6 µg/g E. α–T buvo pagrindinis tokoferolis, nustatytas, tiek HX, tiek CO_2 ekstraktuose, jo kiekis svyravo nuo 61,4 iki 134 µg/g E, gauti duomenys sutampa su literatūros šaltiniais.

3.3.6. *S. virgaurea lapų* ekstraktų antioksidacinis efektyvumas, taikant oksipres ir rancimat metodus

Visi RL 0,5 % ekstrakto priedai tiek rapsų aliejuje (RA), tiek emulsijose (EM) pasižymėjo oksidaciją stabdančiu efektu.

3.6. lentelė. *Solidago virgaurea* L. lapų ekstraktų priedų (0,5%) antiokisdacinis efektyvumas rapsų aliejuje (RA) ir emulsijose (EM) 120 °C temeperatūroje.

	Oksipres 120 °C				Rancimat 120 °C	
Ekstrakto priedai	RA		EM		EM	
	IP	SK	IP	SK	IP	SK
RA (Kontrolėl)	2,18±0,01	1	–	–	–	–
E (Kontrolė)	–		2,64	1	9,24	1
$ETAS_{70}$ HX	1,07±0,03	0,49	–	–	–	–
SKE–CO_2	0,89±0,06	0,41	–	–	–	–
$ETAS_{70}$AC	–	–	2,95±0,00	1,12	9,13±0,01	1,01
$ETAS_{70}$–ET/V	–	–	3,86±0,12	1,46	12,9±0,00	1,40
$ETAS_{140}$–V	–	–	3,05±0,06	1,16	10,7±0,02	1,16

HX–heksanas; AC–acetonas; ET/V–etanolis/vanduo (80/20); V–vanduo; Reikšmės pateiktos, kaip trijų pakartojimų vidurkiai (n=3). IP – indukcinis periodas; SK – stabilumo koeficientas.

SK kito tarp 1,12 ir 1,46 (**3.6 lentelė**). HX ir CO_2 ekstraktai pagreitino oksidacijos procesus, greičiausiai dėl juose buvusių vaškų. EM stabilizavimo efektas mažėjo tokia tvarka: ET/V>V>AC. Didžiausiu AA efektu EM pasižymėjo ET/V ekstraktas (SK 1,46).

IV. IŠVADOS

.

1. Nustatyta, jog tirtų augalų – *B. crassifolia* lapų ir šaknų, *Fagopyrum Esculentum* žiedynų bei *S. Virgaurea* lapų – m frakcijų išeiga priklausė nuo ekstrakcijos temepratūrinio režimo. Didžiausia frakcijų išeiga gauta, taikant pakopinę ekstrakciją organiniais tirpikliais aukštame slėgyje 140 ° C temperatūroje.

2. Tirtų augalų frakcijų antioksidacinis aktyvumas (AA) kito priklausomai nuo ekstrakcijai naudoto organinio tirpiklio bei temperatūrinio režimo.

2.1. Didžiausiu AA pasižymėjo etanoliniai bei acetoniniai tirtų augalų ekstraktai.

2.2. Didžiausias AA aktyvumas gautas, taikant pakopinę ekstrakciją organiniais tirpikliais aukštame slėgyje 140 ° C temperatūroje.

2.3. Visi tirtų augalų lipofilinių frakcijų priedai stabdė oksidacijos procesus rapsų aliejuje bei emulsijose (riebalai/vanduo).

3. Nustatyta, kad pakopinė ekstrakcija, naudojant didėjančio poliškumo tirpiklius, laipsniškai sumažino augalo liekanų AA.

3.1. Pakopinė ekstrakcija tirpikliais aukštame slėgyje 140 ° C efektyviausiai sumažino bergenijos lapų ir šaknų bei grikių žiedynų liekanų AA.

3.2. Kombinuota ekstrakcija taikant superkrizinę ekstrakciją anglies diokisdu bei ekstrakciją organiniais tirpikliais aukštame slėgyje 140 ° C temperatūroje efektyviausiai sumažino rykštenės lapų liekanų AA.

4.1. Nustatyta, jog svarbiausias kiekybinis junginys *B. crassifolia* šaknyse bei lapuose buvo bergeninas. Taip pat nustatytas ženklus kiekis kvercetin–3–β–D–gliukozido. Elago rūgštis aptikta tik lapuose.

4.2. Nustatyta, jog grikių žiedynų poliniuose ekstraktuose, vyraujantys junginiai buvo rutinas bei kvercitrinas. Lipofiliniuose ekstraktuose nustatytas bendras tokoferolių kiekis siekė 1381 μg/g ekstrakte, vyraujantis – α–tokoferolis (260–296 μg/g E).

4.3 Nustatyta, jog kvercitrinas buvo vyraujantis kiekybinis junginys rykštenės lapų ekstraktuose. Taip pat aptikti ženklūs hiperozido, avikularino bei chlorogeno rūgšties kiekiai. Pagrindinis tokoferolis aptiktas lipofiliniuose ekstraktuose – α–tokoferolis (61,4 – 134 μg/g E).

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LIST OF ARTICLE–BASED ON DOCTORAL DISSERTATION

Articles in the journals indexed by Clarivate Analytics Web of Science:

1. **V. Kraujalienė**, A. Pukalskas, P. Kraujalis, P.R., Venskutonis. 2016. Biorefining of *Bergenia crassifolia* L. roots and leaves by high pressure extraction methods and evaluation of antioxidant properties and main phytochemicals in extracts and plant material. *Industrial crops and Products*. Volume 89, pp. 390–398;

2. **V. Kraujalienė,** A. Pukalskas, P.R., Venskutonis, 2017. Multi-stage recovery of phytochemicals from buckwheat (*Fagopyrum esculentum* Moench) flowers by supercritical fluid and pressurized liquid extraction methods. *Industrial crops and Products*. Volume 107, pp. 271–280;

3. **V. Kraujalienė**, A. Pukalskas, P.R., Venskutonis, 2017. Biorefining of goldenrod (*Solidago virgaurea* L.) leaves by supercritical fluid and pressurized liquid extraction and evaluation of antioxidant properties and main phytochemicals in the fractions and plant material. *Journal of Functional Foods.* Volume 37, pp. 200–208.

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1. **V. Kraujalienė**, A. Pukalskas, P.R. Venskutonis. Biorefining of Goldenrod (*Solidago virgaurea* L.) leaf by high pressure extraction and evaluation of antioxidant properties and main phytochemicals in fractions of pant materials. International conference and exhibition on nutraceuticals and functional foods 2016: October 9–13, 2016, Orland, Florida, US. P. 137.

2. **V. Kraujalienė**, P.R. Venskutonis. High pressure fractionation of buckwheat (*Fagopyrum esculentum*) flowers and rod (*Solidago virgaurea*) leaves. Foodbalt – 2015: 10th Baltic conference on food science and technology "Future food innovations, science and technology", ISBN9786090211380. May 21–22, 2015, Kaunas, Lithuania. P. 39.

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